COMPARATIVE STUDY BETWEEN FLUORESCENT MICROSCOPY, MYCOBACTERIAL GROWTH INDICATOR TUBE (MGIT) AND GENE XPERT FOR THE DETECTION OF MYCOBACTERIUM TUBERCULOSIS FROM CLINICAL SUSPECTS OF SMEAR NEGATIVE PULMONARY TUBERCULOSIS AND EXTRA PULMONARY TUBERCULOSIS PATIENTS.

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CERTIFICATE

This is to certify that this dissertation entitled **"COMPARATIVE STUDY BETWEEN FLUORESCENT MICROSCOPY, MYCOBACTERIAL GROWTH INDICATOR TUBE (MGIT) AND GENE XPERT FOR THE DETECTION OF MYCOBACTERIUM TUBERCULOSIS FROM CLINICAL SUSPECTS OF SMEAR NEGATIVE PULMONARY TUBERCULOSIS AND EXTRA PULMONARY TUBERCULOSIS PATIENTS**.

"is the bonafide original work done by **Dr.KAYALVILI.K.K,**Post graduate, under my overall supervision and guidance in the department of Microbiology, Stanley Medical College and Hospital, Chennai, in partial fulfilment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Microbiology (Branch IV).**

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DECLARATION

I, solemnly declare that this dissertation**"COMPARATIVE STUDY BETWEEN FLUORESCENT MICROSCOPY, MYCOBACTERIAL GROWTH INDICATOR TUBE (MGIT) AND GENE XPERT FOR THE DETECTION OF MYCOBACTERIUM TUBERCULOSIS FROM CLINICAL SUSPECTS OF SMEAR NEGATIVE PULMONARY TUBERCULOSIS AND EXTRA PULMONARY TUBERCULOSIS PATIENTS"** is the bonafide work done by me at the Department of Microbiology, Government Stanley Medical College Hospital, Chennai, under the guidance and supervision of **Prof. Dr.R.SELVI, M.D.,**Professor and Head of Department of Microbiology, Government Stanley Medical College, Chennai-600 001.

This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of degree of M.D. Branch IV Microbiologyexaminations to be held in April 2015.

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CONTENTS

COMPARATIVE STUDY BETWEEN FLOURESCENT MICROSCOPY, MYCOBACTERIAL GROWTH INDICATOR TUBE (MGIT) AND GENE XPERT FOR THE DETECTION OF MYCOBACTERIUM TUBERCULOSIS FROM CLINICAL SUSPECTS OF SMEAR NEGATIVE PULMONARY TUBERCULOSIS AND EXTRA PULMONARY TUBERCULOSIS PATIENTS.

Back Ground and Objectives:

Mycobacterium tuberculosis remains one of the most significant causes of death from an infectious agent. The rapid diagnosis of tuberculosis and detection of anti tubercular drug resistance are essential for early disease management.

We determined the performance of the Fluorescent Microscopy, MGIT, Gene Xpert MTB/RIF assay for rapid diagnosis of tuberculosis in smear positive and smear-negative pulmonary and extra pulmonary specimens obtained from clinical and radiological suspects of tuberculosis patients. A total 100 patients , among them specimens such as sputum and BAL fluid were collected from 49 patients, and pleural fluid , ascitic fluid ,Pus, Lymph Node aspirates were collected from 51 patients.

Methods: Specimens were digested and decontaminated with standard NaoH-NALC method for MGIT culture.

Results: Overall ,Modified Ziehl- Neelsen detected 3,Fluorescent Microscopy detected 5, MGIT detected 17 and Gene Xpert detected 26.In smear negative pulmonary specimens MGIT detected 8 strains whereas Gene Xpert 10. Among the

Extra pulmonary specimens ZN detected 3, Fluorescent Microscopy 5, MGIT detected 9, and Gene Xpert 16. There was 100 % agreement between Fluorescent microscopy, in relation to MGIT culture and Gene Xpert. There was 90% agreement between MGIT & Gene X pert with respect to Mycobacterium tuberculosis complex identification.

All 10 MTBC strains were sensitive to streptomycin, Isoniazide, Rifampicin and Ethambutol, which is also sensitive for Rifampicin by Gene Xpert. There was no discrepancy in the sensitivity results. Gene Xpert detected 1 MTBC Rifampicin Resistant strain which was not picked up by MGIT.Among the 7 Atypical mycobacterias all were resistant to Streptomycin, Isoniazide, and Ethambutol ,except one strain which was sensitive to Rifampicin by MGIT.

Conclusion: The results of this study indicated that the implementation of Gene Xpert MTB/RIF assay could dramatically improve the rapid diagnosis of tuberculosis particularly in smear negative pulmonary & extra pulmonary specimens such as pus, lymph node aspirates and wound discharges .Gene X pert negative specimens can be processed in MGIT to recover Atypical Mycobacteria .

Key words: Fluorescent Microscopy, MGIT, Gene Xpert. NALC- N,acetyl-L-cysteine.

INTRODUCTION

"I have no business to live this life if I cannot eradicate this scourge from mankind"- Robert Koch.

(Delivered a lecture at Berlin University after his discovery of tubercle bacilli, on 24 thAugust 1882) .

Tuberculosis is an ancient & highly infectious disease that can potentially involve any organ or system in the body. Tuberculosis remains the major global health problem. It ranks as the second leading cause of infectious disease worldwide[1]. According to WHO report 2012 there are 8.6 million new cases and 1.3 million TB deaths in which, nearly a million among the HIV negative people and 0.3 million among the HIV Co-infected people[1].

Patients having active TB can infect an average of 10 to 15 people annually, it kills 5000 people a day, among which 98% of deaths are in developing countries affecting mostly young adults in their productive age group$_{(35)}$. Higher incidence & Prevalence of tuberculosis cases and deaths occur among men, but the burden of disease among women& children is also high.In 2012 there wasestimated 2.9 million tuberculosis cases & 4 lakh tuberculosis

deaths among women, as well as 5 lakh cases & 74 thousand deaths among childrens[1].

Among the cases, around 5 million were smear negative[35]. Establishing diagnosis in Smear negative pulmonary specimens such as sputum and Bronchio - alveolar lavage fluid constitutes a major problem, becauseof low number of bacilli.

In majority Tuberculosis affects the lungs, with features of persistent cough with or without expectoration, evening rise of temperature, loss of appetite, weight loss, chest pain and hemoptysis[50]. But there are 10 to 15 %&upto 40%[22]of cases with extra pulmonary involvement in developed & low incidence countries[7], but the prevalence is even more higher in developing countries like India with higher incidence of Tuberculosis. Extra pulmonary tuberculosis involves lymph nodes, pleura, bone and joints, abdominal, genitourinary, neurological, laryngeal, meningeal, ocular and serosal membranes[7].

The chance of getting extra pulmonary involvement increases with immunosuppression & in HIV infected patients. Almost 50% of HIV positive individuals is been suffering from extra pulmonary Tuberculosis & the disease progresses rapidly[7].

Atypical clinical presentation of extra pulmonary tuberculosis makes the physician difficult in procuring the clinical samples such as tissue biopsies, synovial fluid aspirates and bone marrow aspirates. Furthermore collection of extra pulmonary specimen often requires invasive procedures and it is not so easy to take further samples.

In developing & resource poor countries like India Acid fast staining (Ziehl-Neelsen) and culture on conventional Lowenstein – Jensen medium forms the main modalities in diagnosing *Mycobacterium tuberculosis*[5] .Since the no of bacilli is less in smear negative & extra pulmonary specimens, only less than 2-5 % of specimens shows positivity by Ziehl-Neelsen staining[11,25],and this makes the microbiologist challenging in getting the yield[7].

WHO has endorsed the implementation of light –emitting diode(LED) auramine fluorescent microscopy & the GeneXpert assay for National Tuberculosis Control Programme inIndia. LED fluorescent microscopy is 6% more sensitive compared to traditional Ziehl-Neelsenstaining[25] .

Although there are variety of newer molecular methods have been introduced in direct detection of *Mycobacterium tuberculosis* complex from clinical specimens rapidly within few hours, culture still represents the gold standard in definitive diagnosis of mycobacterium

tuberculosis[25].However culture is not able to provide rapid diagnosis for clinical management and also it requiresexpensive & sophisticated laboratory facility that may not be affordable by most of the resource limited setting[19].

The main disadvantage of this culture (LJ) method is the delay in time of detection. As the tubercle bacilli is a slow growing organism, it takes more than a month to grow and only very few strains grow faster[5]. The mean time for the detection of culture of Mycobacterium tuberculosis on LJ medium is 31±9 days[35].

So it is obligatory to device a rapid & reliable diagnostic culturemethod in detection of *mycobacterium tuberculosis*. The MGIT system Mycobacterial Growth Indicator Tube, which is a broth based liquid culture medium , introduced in 1980 has considerably improved the mean time of detection by 18±14 days[35]. This method is very easy to use, noninvasive, and non-radiometric, not labour intensive and so can be executed in any laboratory.

The Xpert MTB/RIF is a fully automated Catridge based Nucleic Acid Amplification test with an sensitivity of five genome copies of purified DNA and also it can detect more than 99.5% rifampicin resistance mutations ,an indicator of multidrug resistance tuberculosis in less than 2 hours. This requires only minimal biosafety infrastructure and training[22].

Tertiary care hospitals like Medical college hospitals has the maximum referral of Extra pulmonary cases for establishing the diagnosis. Extra Pulmonary cases of Stanley Medical College from 2011-2013 statistics includes 2,379 cases. Because of high case load it is necessary to diagnose rapidly and to give the anti tuberculous sensitivity as soon as possible to start the treatment early and to prevent the complications Multidrug resistant tuberculosis pose a major public health problem. The magnitude of which is higher about 2.2 % [25].

Conventional methods of isolation although specific, take 4-6 weeks time and have variable sensitivity depending on the type of specimen. Susceptibility tests resulttakes another 3-4 weeks time[35]. But still the information about the performance of LED fluorescent microscopy &Xpert assay for diagnosing Extra pulmonary & smear negative tuberculosis is less compared to pulmonary tuberculosis

The purpose of this study is to test& compare the efficiency and reliability of LED FLUORESCENT MICROSCOPY, liquid culture medium(MGIT), & GENE X pert for the detection of Mycobacterium tuberculosisbacteria in smear negative pulmonary specimens and extra pulmonary specimens, and to do anti tuberculous sensitivity pattern by Mycobacterial Growth Indicator Tube.

AIMS AND OBJECTIVES

1. Isolation of Mycobacterium tuberculosis from smears negative pulmonary Tuberculosis and Extra pulmonary Tuberculosis patients by MGIT – Mycobacterial Growth Indicator Tube, Fluorescent Microscopy & Gene Xpert.
2. To compare the positivity in modified Ziehl- Neelsen staining & Fluorescent staining.
3. Comparison of positivity between MGIT – Mycobacterial Growth Indicator Tube and Gene X pert.
4. To test the antitubercular drug sensitivity by MGIT.
5. To compare the antitubercular drugsensitivity by MGIT and Gene Xpert.

REVIEW OF LITERATURE

History of Tuberculosis

Tuberculosis is an ancient disease caused by bacillus *Mycobacteriumtuberculosis.* Genus *Mycobacterium* was originated more than 150 million years ago. *Gutierrez* and her colleagues said that, the early progenitor of M. tuberculosis was present in East Africa as early as 3 million years ago, and they concluded that it must have infected early hominids at that time[4].

The ancestral home of tubercle bacilli was Eastern parts of Africa. In Egypt this disease was evident more than 5000 years ago. Typical skeletal abnormalities , such as characteristic pottsspine deformities, have been found in Egyptian mummies and were also depicted in early Egyptian art[4]. M. tuberculosis DNA had been amplified from tissues of Egyptian mummies. TBspondylitisfirst description was written in Sanskrit between 1500 & 700 BC[2].

With the revolution in urbanization & proximity of living the epidemic , described as Great white plague. The incidence has increased from mid 1700s following successive pandemic and has spread throughout Europewith the peak incidence in 1800s.

Tuberculosis was noted in the Biblical books of Deuteronomy and Leviticus, by the Hebrew word schachepheth

.Tuberculosis is noted in India and China coined as early as 3300 yrs and 2300 years ago one-to-one. As in Egypt, archeological proofof tuberculosis is found in America. Bony tuberculosis, such as Potts disease is demonstrated in Peruvian mummies.

In Greece Tuberculosis was baptized as phthisis. Hippocrates clearly portrayed and understood the clinical presentation of tuberculosis. Phthisis attacks between the ages of 13 and 35, it was inscribed in his aphorisms, clearly identifying the predilection of young adults. Laennec, clearly described the pathogenesis of tuberculosis and amalgamated the concept of the disease, whether pulmonary or extrapulmonary.[4]

French surgeon Jean-Antoine Villeminconvincingly proved the infectious nature of tuberculosis, in 1865 after he inoculated a rabbit with a little amount of purulent liquid from a tuberculous cavity detached at autopsy from an individual who died of tuberculosis.

In his presentation, Koch presented demonstrations of the tubercle bacillus, his famous postulates, perhaps called the Koch-Henle postulates, which is set as the standard for the parade of infectious etiology.

RESEARCH JOURNEY OF TUBERCULOSIS

In 1890 Koch invented Tuberculin , & he injected himself & he perceived that he developed an unusual ferocious attack of ague… and rise of body temperature & he clinched this might be of use diagnostically.

In 1907 clemensFreiherr von Pirequet noted that positive tuberculin testing reflected latent tuberculosis.

In 1908 Charles Mantoux introduced the use of cannulated needle & syringe to inject intradermally.

IN 1930 Florence Seibert developed Purified protein derivative.

1859 Herman Brehmer opened his Heilenstat in Silesian Mountain village of Gobersdorf where he emphasized a regimen of REST,A RICH DIET,CAREFULLY SUPERVISED EXERCISE. This sanatorium is generally considered the first such facility devoted to the treatment of tuberculosis.

Fads and fancies have gathered about so called OPEN AIR TREATMENT.

In 1921 Calmette and his associate CamilleGuerine undertook a herculean effort to attenuate M.bovis for use as vaccine.

Acid-fast stain, was first defined by two German doctors: the bacteriologist FranzZiehl and the pathologist Friedrich Neelsen

During World war I there was a high re-emergence. Initially TB suspects were screened with X ray alone, and then with tuberculin testing followed by BCG vaccination. In 1974 WHO discouraged both radiographic screening & promoted sputum microscopy of symptomatic individuals .

PAS- Para amino salicylic acid by Gerhard Domagk &thiosemicarbamazone yielded the first therapeutic efficacy agents .

Isoniazid was the first oral mycobactericidal drug in 1952 and rifamycins in 1957 was invented[4].

Today Tuberculosis remains endemic in many of the countries like India. Several factors added to these drifts ,such as underlying diseases, including diabetes, chronic renal failure, chronic obstructive pulmonary disease, cirrhosis of liver, blood leukemias& lymphomas . Social factors includes, over crowding, poor nutrition, & migration from countries with a high prevalence(2

EPIDEMIOLOGY

Tuberculosis remains most important global health problem. It affects & kills millions of people each year, and it stands the second leading

cause of death from amongst the infectious diseases world wide, after HIV.The Asia and Western Pacific region countries accounts 58% of worlds TB cases. INDIA has the leading number of TB cases that is 26% of the global total. According to WHO global health report 2012 there were 8.6 million new TB cases & 1.3 million TB deaths in which cause 1 million deaths among the non HIV people and 0.3 million among the HIV positives.[1]

The burden among females and children is also higher, there are 2.9 million cases & 410,000 Tb mortality among women in which 250,000 among HIV negative & 160,000 among HIV positive women, and estimated 530,000(6%) cases & 74,000 deaths among childrens[1] .Globally TB death rate dropped by 45% since 1990 & TB incidence rate is also falling in most of the countries

In INDIA incidence of non HIV TB patients was 2.4 million , prevalence was 3.9 million & HIV positive incident TB cases was 1,40,000.

Between the years of 2008& 2013 in INDIA the highest mortality rate was 390,000 & the lowest was 170,000 in the HIV negative individuals, & in the HIV positive patients highest TB mortality rate was 48,000 & the lowest rate was 37,000.

Among the 8.6 million incident TB cases around 1.1 million patients are co-infected with HIV[1]. In majority Tuberculosis affects the lungs, but there are 10 to 15 % upto 40%[22] of cases with extra pulmonary involvement in developed & low incidence countries[7]. The prevalence is even more in developing countries like India with higher incidence of Tuberculosis.

Mycobacterium Tuberculosis BELONGS TO

ORDER – Actinomycetales

GENUS – Mycobacterium

FAMILY – Mycobacteriaceae

GENERAL CHARACTERISTICS

Mycobacterias are non-motile,nonspore forming, weakly Gram positive ,aerobic or microaerophilic, straight or slightly curved rod (0.2-0.6x1.0 – 10 μm) Sometimes shows coccobacillary or branched ,or Filamentous form can also be seen ,on slight strife it is fragmented into rods or coccoid elements.

PATHOGENESIS:

In > 90% of patients infected with M.tuberculosis, the bacteria is in latent infection. For active disease to occur risk of infection is 5% in

the first 18 months and 5% for the residual life.[3],Roughly 2 billion people living worldwide is having latent state.

Tuberculosis is seen in 2 forms, either from a recent infection(new exogenous infection) or from a reactivation of dormant tubercle bacilli(old endogenous disease)[2].The annual risk of getting pulmonary TB following recent primary infection is 300 times more than the reactivation.[2] In young adults individuals disease is mostly due to new exogenous infection and in elderly , due to reactivation.

MANIFESTATION OF TUBERCULOSIS

Pulmonary TB is commonest form because of inhalational(aerosol) spread . Bacteria reaches the blood via the lympho - hematogenous route & it coud affect any organ. Because of HIV ,incidence of extrapulmonary TB is also increasing .

PULMONARY TUBERCULOSIS:

It is divided into primary and post primary (reactivation)TB

PRIMARY TB –accounts for 23 to 34% of adult TB cases[2]

Following inhalation of a droplet aerosol containing bacilli ,it come in the terminal alveolus & resulting primary parenchymal (Ghon) focus,drains via the local lymphatics to the regional lymph nodes.Combination of Ghonfocus + lymphangitis + regional lymph

node involvement is called Ranke complex,then the disease extends via bronchially or hematogenously.

This manifestradiologically as parenchymal disease, lymphadenopathy ,pleural effusion, miliary disease or atelectasis.

POST PRIMARY TB:

Results from reactivation of dormant primary infection in > 90 % of cases[2].

This occurs as result of very poor nutrition status, HIV infection, cancers, ageing all are associated with high morbidity and mortality. Parenchymal disease may demonstrate thecaseous and liquefaction necrosis & communicate with trachea-bronchial tree to form cavities. Bilateral upper lobe involvement is most common.Endobronchogenic spread is characterized by multiple micronodules distributed at a distant from the cavity site. If left untreated at the end stage it leads to lobar or complete collapse&opacification. Fibrosis can also occur(2).

EXTRA PULMONARY TUBERCULOSIS

Extra pulmonary involvement results from lymphogenous spread or hematogenous spread ,frequently a pulmonary focus of infection.Extra pulmonary TB constitute about 15 – 20 % of all cases of TB. In HIV positive patients it accounts more than > 50 % of cases of TB[6,7] . Extra

pulmonary tuberculosis involves lymph nodes, pleura, bone and joints, abdominal, neurological, laryngeal, meningeal, ocular and serosal membranes[3].Lymphadienitis , most common form of EPTB followed by pleural effusion .Additionally atypical presentation of Extra pulmonary TB ,less number of tubercle bacilli present in the specimen makes the diagnosis difficult. Attaining samples is also tough from the EPTB cases.

CLINICAL FEATURES.:

Pulmonary Tuberculosis – chronic cough, sputum production, loss of apetite, weight loss, fever, evening rise of temperature, night sweats and Hemoptysis.

Extrapulmonary TB – patients presents with the symptoms according to the organ or system involved[3].

LAB DIAGNOSIS OF MYCOBACTERIUM.

Various methods available for diagnosis of TB includes :

1.Smear microscopy.

2.Culture on LJ,MGIT,BACTEC.

3.Serological diagnosis.

4.Nucleic Acid Amplification methods- PCR,Gene X pert, Line probe assay,LAMP.

Pulmonary Tuberculosis :

Before the start of treatment precise diagnosis is obligatory. Sputum smear microscopy forms the core diagnostic practice in most laboratories especially in developing countries like INDIA. The bacilli in the sputum can be detected by ZN stain &fluorochromestain,but there is a substantial statistical difference in the sensitivity of two methods has been reported[45]. ZN staining has very low sensitivity & specificity chiefly in extra pulmonary cases,only 2% [11].In PTB after concentrating the sputum by petroffs method the sensitivity was increased about 44.1% for ZN staining & 71.6% for Auramine O staining [29,30,]IIgazli et al stated smear positivity of 4.2% in EPTB[6].Smear positivity of LED microscopy in EPTB was 9.1% and the specificity was 95%.[25].Using a molecular technique , transmission of smear negative patients , to smear positive patients is 22%. As per WHO statistics 2004 out of 8.6 million TB cases around 5 million were smear negative(35).

Actually dissimilar results were attained by Jain A et al ZN 32.7%, AO 41.6%, Githui et al ZN 65%, AO 80%, Besides ZN could detect bacilli only when there was 10^5 bacilli per ml of sputum but AO stain can detect 10^4bacilli per ml of sputum[45]. Infection with HIV is known to amend the presentation of pulmonary tuberculosis. Fluorochrome

staining was 45% more capable when compared to ZN staining (29%) in detecting cases with HIV seropositivity, particularly paucibacillary cases[27].

In the early stages of HIV infection, when cell mediated immunity is only partly suppressed , pulmonary tuberculosis presents typically as upper lobe infiltrates and cavitation with high bacillary load in the sputum, however in the late stages, primary tuberculosis, with diffuse interstitial and miliary infiltrates, no cavitation is appreciated resulting in paucibacillary picture of sputum. In the present situation withgrowing incidence of HIV a speedy and highly sensitive method which can detect paucibacillary cases is the supreme one[27].sputum positivity was less in HIV patients compared to HIV negative patients. Smear negative pulmonary tuberculosis is responsible for 17% of TB transmission[27]. Especially in CSF the sensitivity of ZN staining is less only 20% [28].

CULTURE:

Culturing Mycobacterium tuberculosis is the gold standard investigating modality. Can be cultured in both solid media and liquid media .

SOLID MEDIUMS

Egg based –(Lowenstein-Jensen(LJ),LJ Gruft, LJ with pyruvic acid,LJ with iron,petragnani,American Thoracic Society medium,petran-LJ with antibiotics.)

Agar-Based – a)Middlebrook 7H10 &Middlebrook 7H10 selective.

b)Middlebrook 7H11 &Middlebrook 7H11 selective(Mitchisons)

c)Middlebrookbiplate(7H10/7H11S agar).

LIQUID MEDIUMS

BACTEC 12B medium.

Middlebrook 7H9 broth.

Septichek AFB.

Mycobacterial Growth Indicator Tube.

For prime recovery of mycobacteria, a minimum of two one liquid medium and anothersolid medium is recommended.

SOLID CULTURE:

The advantage of solid media over liquid media is characteristic colony morphology,pigmentation, worthy growth from small inocula, & low rate of contamination. Atleast minimum of two solid culture medias should be used & all the specimens should be processed that is

digestion ,decontamination and concentration of mycobacteria should have been done[49]. Superlative method for concentrating the specimen is NaOH-NALC digestion decontamination technique[35,36,37]. Moreover 0.2 ml of processed specimen should be inoculatedand incubated ideally at 37^0C under 5-10% CO_2 atmosphere and with high humidity. For the first week cap should be slightly loosened, till the excess water to be evaporated, and for the entry of CO_2 & should be incubated in a slant position.

Then the culture tubes should be examined for growth every week[49]. Contaminated cultures should be discarded & to be reported as CONTAMINATED unable to detect mycobacteria, & additional specimen should be requested. Second specimen must be cultered again after improved decontamination. Most of the isolates starts growing between 3 and 6 weeks, few isolates appears after 7 weeks of incubation. When growth occurs colony morphology, growth rate& pigmentation shoud be noted. After 8 weeks slants with no growth should be reported as negative cultures & discarded.

LJ detected only 53.3% of cultures detected by liquid cultures, the mean time of detection of positive cultures were 31±9.4 days[35].The mean time of detection of LJ in Smear negative specimens according to Badak&Pffffer et al was 31 and 27.2 days respectively. Badak et al

who compared between LJ, MGIT & found LJ culture sensitivity from smear negative specimen was 58.8% . But the sensitivity is higher if the cultures were done from the smear positive specimens. In smear positive specimens the sensitivities of LJ medium was higher 93.5%,85.7% according to Badak&Pfyffer studies respectively[35].

Extra pulmonary TB are often smear negative which makes difficulty in diagnosis. The rate of detection of LJ in extra pulmonary cases was only 58%[11].The comtamination rate was 8% in LJ ,the advantage of sold medium over liquid medium is less contamination rate[11].

MIDDLEBROOKS SOLID MEDIUM

This media contains defined salts,organiccemicals, agar, albumin,biotin&catalase.

It is transparent allows early detection of growth after 10-12 days compared to LJ. Only difference between Middlebrook 7H10& 7H11 is the later contains 0.1% casein hydrolysate which improves the rate & amount of mycobacteria resistant to Isoniazid[50]

SELECTIVE MIDDLEBROOKS SOLID MEDIUM

Modification of Middlebrooks medium is addition of antibiotics to 7H10 & 7H11 medium which makes it as a selective medium and

drops the contamination rate. Mycobacteria grows best at 3-11% CO_2atmosphere ,if this selective medium is used[(50)].

MICRO COLONY DETECTION ON SOLID MEDIA

- Middle brooks 7H11agar solid medium plates are inoculated , incubated and examined microscopically every alternate days . In 7 – 10 days micro colonies of mycobacterium can be detected , it is less expensive , less time consuming than the LJ culture , but it appeared to be less efficient [10,34,50.]

- **LIQUID CULTURES:**

MYCOBACTERIAL GROWTH INDICATOR TUBE (MICRO MGIT)

Round bottomed glass tube contains Middlebrooks 7H11 broth base - 7 ml ,a fluorescent compound is implanted in silicone on the base of the tube. This fluorescence substance is sensitive to the dissolved oxygen in the liquid medium ,& if O_2 is there it quenches the light. As the multiplying bacteria utilizes all the oxygen the fluorescence is unmasked & it will be visible to be detected by UV light. Growth is also seen as a nonhomogenous turbidity or very small grain of flakes to be seen by naked eyes [50].After adding supplements such as (OADC & PANTA) with sterile aseptic precautions , in to tube about 0.5 ml

of processed specimen using NaOH – NALC mixture is added into the tube & to be & incubated @ 37^0 C .Tubes to be incubated minimum of 6 weeks, & start reading the tubes from 2 nd day onwards. The positive tube should be confirmed for AFB preferebly by ZN staining &subculturing on BAP[8,8,36,50].

The recovery rate of MGIT ranged from 65% to 96[8] especially in EPTB specimens. The sensitivity was 91.6% , TTD was 7-10 days for smear positive & 8-44 days for smear negative specimens. The use of L-J medium in combinationliquid media increased the positivity to 93.3%

MYCOBACTERIAL GROWTH INDICATOR TUBE (MGIT 960)

This system is, non-invasive, non- radiometric,easy to use, fully automated with high enactment. Advantage over manual MGIT is, it itself can incubate & monitor for every 60 min for the growth by fluorescence. There is no considerable difference in the sensitivities between the manual MICRO MGIT & automated MGIT[10].

Sensitivity of MGIT960 for smear-negative specimens 75%. By combining solid & liquid medium positivity increases. MGIT960 was found to be positive in 98.% cases with average time to detect growth around 9.6 days ranges from (2-39 days) ,and that of LJ 63.9% sensitive with average TTD of 28.8 days (6-48 days)and of LJ

alone 1.93% . Isolation rates of Mycobacteria in pulmonary and extrapulmonary samples were 61.83% and 21.98% by M960, 44.01% and 4.96% by LJ, respectively[35,36]. The recovery time was lesser for smear-positive specimens (averages, 11.2 days with the MGIT 960 system, 13.6 days, and 22.9 days with Lowenstein - Jensen medium) than among smear - negative specimens (18.4, and 32.3days, respectively)[42].

BACTEC 460 AFB SYSTEM

This instrument embraces a scintillation counter, which consists of needle aspiration assembly, which is a movable track up to 60 bottles can be occupied. As each culture bottle in the movable track comes beneath the needle assembly, the needle is depressed through the rubber stop and the head gas is removed to get delivered to the scintillation counter which , is replaced with fresh 10% CO2, before the next bottle is sampled the needle is sterilized by electrically.The BACTEC 12b 20 ml broth bottle contains 4ml of broth culture medium consisting of Middlebrook 7H9 broth base;bovine serum albumin;casein hydrolysate'catalase and polyoxylene stearate with antibiotics and ^{14}C labeled palmitic acid as growth indicator.0.5 ml of the concentrated & processed specimen is injected and if mycobacteria is present in the specimen $^{14}CO_2$ is released into the head space which

is picked up by the detector system and is converted into growth index.

The mean times to detection of mycobacteria in smear negative patients were 12, days in BACTEC460. TheTTD was lesser for BACTEC 460 than MGIT tube, and the difference was not significant . The TTD was shorter for MGIT than L-J, and the difference was highly significant[35] .

SEPTI – CHECK AFB SYSTEM.

- This method allows identification of MTB complex and MOTT (atypical mycobacteria) simultaneously contains Middlebrook 7H9 broth with CO2 and paddle with agar media inside a plastic contains non selective middlebrook 7H11 agar , and on the other side with 2 divided agar portions , one half for CA (chocolate agar) which allows detection of contamination and the other half contains 7H11 agar with NAP - Para - nitro - a -acetylamino - b - hydroxyl-propiophenone , with which can detect & differentiates MTB complex and MOTT.

- . This method allows identification of mycobacteria from both pulmonary and extrapulmonary specimens. A Study conducted in USA shown that this method gives better result compared to BACTEC 460 TB System[10.34.50]

- Isenberg and coworkers have found & said that this septi-check system was more sensitive than LJ,7H11 and BACTEC broth . They concluded that better recovery could be endorsed due to biphasic nature of the systems recurrent exposure of agar media to proliferating organisms in broth.

ESPII BLOOD CULTURE SYSTEM

This is designed to determine consumption and/or production of gas by organisms , helps in getting the yield of mycobacteria from blood culture bottle is placed in an incubation module attached to sensor , having plastic housing, recessed needle and hydrophic membrane, Significant pressure change signated early from the consumption of O2.

Williams Bouyer et al compared ESP culture system II positivity rate was 71% for ESP II . The TTD was 17 days for ESP II and 12 for MGIT [10,34,50]

The BACTEC Myco/F LYTIC

- This culture bottle contains a lytic agent to release the mycobacteria that have been phagocytosed and present inside the WBCs'..

The sensitivity was 81% and the mean time of detection was 15.3 days[34,50]

MB / BacT , MYCOBACTERIA DETECTION SYSTEM

This sytem is designed similar as BacT/Alert blood culture system. This bottles contains Middlebrooks 7H9 broth, CO_2 ,nitrogen and O_2undervaccum Mycobacterial Growth supplements and PANTA antibiotic mixture reconstituted ,& added. Add 0.5 ml of specimen after decontamination , base of bottle contains gas permeable sensor changes from green to yellow color when CO_2 is produced by multiplying bacteria.

IDENTIFICATION OF MYCOBACTERIAL SPECIES FROM THE CULTURE POSITIVE

1) **Biochemical typing**

   i. Niacin Accumulation

   ii. Nitrate Reduction

   iii. Tween 80 Hydrolysis

   iv. Catalase Activity

   v. Aryl sulfatase Activity

   vi. Urease Activity

   vii. Pyrazinamidase

2) LIPID ANALYSIS :

- By gas chromatography , mass spectrum and high pressure liquid chromatography (HPLC) (10,50)
- HPLC

3) PROBE BASED IDENTIFICATION

- Peptide nucleic acid (PNA) FISH
- PNA is a novel DNA in which sugar phosphate backbone of DNA has been replaced by polyamide.

4)SHERLOCK MYCOBACTERIAL IDENTIFICATION SYSTEM(SMIS)

It is based on the mycolic pattern generated by HPLC(HIGH PERFORMANCE LIQUID CHROMATOGRAPHY), it uses computerized software for mycobacterial detection.

5)PCR RESTRICTION ENZYME ANALYSIS

It exploits 16srRNA gene for & 65 KDahspgene for identification.

6)DNA CHIPS

It involves oligonucleotide arrays or DNA chips, which is designed to determine the specific nucleotide sequence diversity 16srRNA &rpo B gene for identification.

DETECTION OF MYCOBACTERIA DIRECTLY FROM CLINICAL SAMPLES DIRECTLY

1) GENOTYPIC METHODS

2) PHENOTYPIC METHODS

GENOTYPIC METHODS (PCR)

- Allows amplification of DNA even when there was only few copies , it enables rapid visualisation and identification

  The target sequence is IS6110, this deals numerous targets for amplification because it is present up to 20 times in the genome.

  In Bangladesh, a study stated the sensitivity and specificity of PCR using IS6110 was 94.74% and 100 % against culture [10,34]

TMA (Transcription mediated amplification) and Nucleic Acid Amplification Technique

Amplified mycobacterial direct test

- This recognizes MTB complex directly from specimen rapidly discriminates from NTM (The sensitivity and specificity against cultures on smear positive specimen was 96% and 100%.

GENEXpert MTB/RIF ASSAY

Automated molecular NAAT, detects MTB complex and also resistance to rifampin. It is a hemi rested real time PCR assay, amplifies sequence of rpo B gene , then probed with molecular beacons for detecting mutations within the Rifampicin resistance determining region and detects within 2 hours. The GeneXpert MTB/RIF assay is a , integrated device which doessample processing and real-time PCR analysis in a single hands-free step inclinicalspecimens directly[20,21,22] In multi-centre studies, a single MTB/RIF test detected 100% of smear-positive TB patients and about 3/4 of the smear-negative TB patients& also recognized rifampicin resistance, thus identifying patients who are in need of second line drug treatment(21). This assay test has intermediate sensitivity which means better than smear microscopy but lesser than broth culture prone for false-negative results (20). MTB/RIF test is a major

improvement in TB diagnosis, but has some restrictions, including the limited diagnostic cartridges, contineous electricity supply, & the need for annual servicing and calibration .Among the estimated 500,000 people who develope MDRTBanually,only 7% are diagnosed(20).

The sensitivity and specificity of the test were 82% and 100%, respectively for pulmonary specimens. The sensitivities were 100% for smear positive and 68% for smear negative specimens. For extrapulmonary samples, the sensitivity was 52% and specificity was 100%. The sensitivities for smear positive and smear negative extrapulmonary specimens were 100% and 37%, respectively 90% of RIF resistant isolates also exhibit resistance to isoniazid (INH)[21] Therefore, the detection of RMP resistance may serve as a marker for MDR TB. The Xpert MTB/RIF assay appropriately identified rifampin resistance in 99.1% of patients (21)

LINE PROBE ASSAY

Nucleic Acid Amplification techniques which are available today allows the prediction of drug resistance in clinical specimens within one working day. One such molecular technique, GenoType Mycobacteria Drug Resistance (MTBDR) is a commercial assay, uses a DNA strip technology and a reverse hybridisation method for a line probe assay. The MTBDR assay , introduced in 2004 identifies

mutations in the rpoB gene for rifampicin resistance , as well as mutations in the katG gene for high‐level Isoniazid resistance. The GenoType MTBDR plus, the second generation assay new ones also detects mutations in the inhA gene that confers resistance to low‐levels of $INH_{(52)}$

. However, proper laboratory design, standard biosafety practices and quality control to avoid cross‐contamination are required for the MTBDR, which is similar to other nucleic acid amplification assays. The BACTEC DST system is cheaper than the MTBDR it is not easy to perform requiring high standards of biosafety. The use of radioactive materials, & the need for disposal of radioactive waste, represented the major disadvantage of this system. Many previous studies have also found that sensitivity (81‐99%) and specificity (94‐100%) of MTBDR assay for the detection of RIF resistance is high and the most common mutation area is codon 531 on the rpoB gene region

OTHER MODIFICATIONS OF PCR

- STRAND DISPLACEMENT AMPLIFICATION (SDA)
- NUCLEIC ACID SEQUENCE BASED AMPLIFIACTION (NASBA)
- BRANCHED DNA (b-DNA)

- LIGASE CHAIN REACTION
- LINE PROBE ASSAY (LIPA)

Targets in different methods of PCR

Target was IS6110 & 16sRNA

- Spacer oligonucleotide typing (Spoligotyping)
- RFLP using IS6110
- MIRU (Mycobacterial interspersed repetitive unit)PHENOTYPIC METHOD

FAST Plaque TB™

- This uses mycobacteriophage to detect presence of MTB from the sputum specimens, it is a rapid, easy to perform, manual test.

SEROLOGICAL DIAGNOSIS OF TUBERCULOSIS:

ANTIGEN DETECTION ASSAYS FROM CLINICAL SAMPLES

1. Capture ELISA- a quantitative test which detects LAM(lipoarabinomannan) from clinical samples such as urine ,sputum & also extra pulmonary samples. sensitivity& specificity is 93% & 95% .
2. Antigen detection in fluids by ELISA, Latex Agglutination & Reverse passive haemagglutination test - This can detect free

mycobacterial antigen from CSF, Pleural fluid ,at the concentration of 3-20ng/ml glycolipids,mycobacterialsonicates. The sensitivity & specificity of the these tests ranges from 40-50% & 80-95%.

INDIRECT APPROACH

Antibodies to mycobacterial antigens in sera are detected by either polyclonal or monoclonal antibodies. False positives can occur.

1.TB STAT-PAK – Immunochromatographictest,can discriminate between dormant or active TB in plasma, serum & whole blood. Its sensitivity & specificity is yet to be ascertained in countries like INDIA.

2.ENZYME IMMUNOASSAY FOR DETECTION OF ANTIMYCOBACTERIAL SUPEROXIDE DISMUTASE ANTIBODY- It is less sensitive in INDIA because the PPV is 77-88%

3.INSTA TEST TB- It enables antibody detection from blood or serum.Commercially available tests are

a.MycoDot^{TM}(Dot-blot) using LAM antigen.

b.Detect-TB(ELISA) using Recombinant protein peptide.

c.PathozymeMyco (ELISA) using 38kDa Ag & LAM.

MISCELLANEOUS DIAGNOSTIC METHODS

1.TB MPB 64 patch test- This is a specific antigen of M.tuberculosiscomplex.Test becomes positive in 3 to 4 days after patch application. specificity is 100% & the sensitivity is 98.1%.

2.IFN gamma PRODUCING CELL MEASUREMENT- The ESAT 6,A SPECIFIC Ag ,which induces T cells of TB patients to secrete IFN gamma.The level of IFN gamma increases in treated & untreated patients ,helps in monitoring the therapy of TB patients.

ANTITUBERCULAR SENSITIVITY

For initial empiric treatment 4-drug regimen: isoniazid, rifampin, pyrazinamide, and either ethambutol or streptomycin. The specimen should be sent for anti tuberculous sensitivity testing.

Antimicrobial Susceptibility Testing AST can be performed by the proportion method using LJ medium and also by automated method by BacT Alert 3D system.

a. LJ Proportion Method :

The bacterial suspension is prepared by picking 2-3loopful of colonies into 0.2ml sterile DW in a screw capped bottle (approximately 4mg weight of the growth in LJ medium). The

colonies on the drug free medium isexpressed as a percentage. 1% or more of growth is considered as resistant for all drugs[58]

b.BacT Alert 3D Proportion Method :

The BacT/Alert MP bottle containing the growth of Mycobacterium tuberculosis (≤36 hours, subculture, then the growth was diluted as 1:1 in sterile DW). This forms the direct growth control of McFarland no.2 .This is injected into all drug containing and control bottles. A 100-fold dilution of the DGC (0.1ml of DGC + 9.9ml of SDW) is prepared and 0.5ml is added to another BacT/ Alert MP bottle and this coostitutes the 1% growth control (1% GC). These bottles should be incubated in the system 12 days at 35^0c and monitored to detect the growth. An isolate is considered as resistant if the drug containing bottles flagged positive at the same time or just before the control tube. An isolate was considered susceptible if the drug containing bottle remained negative during the whole test period or flagged positive after the control tube. If the DGC did not flag positive in 12 days the test was invalidated and it should be repeated[58,16,18]

The average time of detection for resistance by the LJ proportion method ranges from 18-32 days withaverage of 20 days. The drug resistant isolates takes ≥10- 12 days by BACTEC and 27-32 days by LJ method to grow which, was longer than the sensitive isolates.

Theresistance detected by LJ which is the 'gold standard' for comparison, is in the order of least effective being isoniazid (44.6%) ,then ethambutol (17.4%) and rifampcin . Mono drug resistance was found in (21.5%) isolates, Isoniazid was the least effective single agent[58]. Resistance to 2 drugs is frequently observed with INH and rifampicin (15.7%), 3 drug resistance was among INH, rifampicin andethambutol, resistance to >3 drugs was seen in 8.3% cases

MDRTB was 1.7 times morecommon in pulmonary isolates , compared to extra pulmonary using both methods. Among all the second line drugs maximum resistance was seen in Kanamycin (7.4% 100% agreement was observed for Streptomycin, Amikacin, Capreomycin , Rifampicin, Ethionamide, Ciprofloxacin and Kanamycin. 91.5% agreement for Isonizaid, 85% for pyrazinamide and 72.4% for Ethambutol is seen in a study from DELHI$_{(58)}$.

As recommended by the IUATLD (2001) sensitivity, specificity, (PPV) and (NPV) of each drugs for the colorimetric BACTEC system were determined as compared with LJ method. The sensitivity was 100% for all the first and second-line drugs. It was lower for Ethambutol, 68%,and 87% for Pyrazinamide and 91.5% for Isoniazid.

In present study lower sensitivity was observed for pyrazinamide (98.3%), isoniazid (95%) and ethambutol (94.7%). 100% sensitivity

and specificity was reported for Rifampcin, followed by INH and pyrazinamide although streptomycin and ethambutol gave lower sensitivity and specificity [12]. Angeby KAK et al. have reported 100% specificity to all first line drugs and 100% sensitivity for ethambutol followed by 96% for isonizaid, 92% for rifampcin and 78% for streptomycin .Barreto et al. reported 100% sensitivity and specificities for rifampicin and ofloxacin(58)

MYCOBACTERIAL GROWTH INDICATOR TUBE FOR ANTIBIOTIC SENSITIVITY

One of the promising & accepted method in various settings is the fluorimetry based liquid culture detection system, Mycobacterial Growth Indicator Tube (MGIT 960) . Reports of "false resistance" to INH at a concentration of 0.1 µg/ml recommended by the manufacturer haddocumented(16). MGIT tubes supplemented with 0.8 ml of supplement (MGIT 960 SIRE supplement; Becton Dickinson) is inoculated with 0.1 ml of the drug solution and 0.5 ml of the test strain suspension. For preparation of the drug-free growth control tube, the organism suspension was diluted 1:100 with sterile saline, and then 0.5 ml was inoculated into the tube (proportion testing[16,17,18]. In general, the MGIT 960 system tends to give resistant test results more frequently than the Bactec 460 system.

The results indicated high sensitivity (>91%), specificity (>95%) and accuracy (>92%) for detection of INH resistance by MGIT 960 in comparison to conventional MIC methods. Comparison of MGIT 960 with phenotypic methods..Use of MGIT 960 in routine has significantly reduced the time for detection of resistance thus giving way for early and accurate diagnosis(16.17)

Multi Drug Resistant Tuberculosis

The multi-drug resistant TB (MDR-TB) is defined as resistance to isoniazid and rifampicin in the presence or absence of resistance to other drug. Data from various studies conducted by Tuberculosis Research Centre , have found to have levels ranges from 1% to 3% in new cases and about 12% in re-treatment cases. Multi Drug resistant tuberculosis is frequently been encountered in India and its presence is known virtually from the time drugs were introduced for the treatment [3,4]. Six- months curative treatment for TB costs less , but MDRTB treatment costs more. Additionally, MDRTB management requires skills and specialist drug supply that currently have restricted availability in the low-income countries where most cases occurs. The early diagnosis of drug-resistant TB iscost-effective , but risk factors for TB drug resistance have very poor predictive value, especially in higher prevalence settings [20]. Much consideration has focused on the

burden of (MDR) TB, i.e., resistance to the first- line drugs and the emergence of extensively drug resistant TB. The rise of resistant TB and the increased spread to human population , due to coinfection with HIV virus are driving the worldwide TB pandemic and will worsen the situation in the years ahead, with devastating effects in poor countries, whose economies suffer most from this development[17].

MATERIALS AND METHODS

STUDY DESIGN:

Prospective study.

PLACE OF STUDY:

1. Department of Microbiology, Stanley Medical College, Chennai.

2. Department of TB and Chest Medicine, Stanley Medical College, Chennai

STUDY POPULATION:

During this study period, patients with complaints of cough with expectoration & fever for more than 2 weeks , and also patients with complaints of suspected extra pulmonary tuberculosis were also included in the study.

INCLUSION CRITERIA:

Outpatients and inpatients with clinical symptoms suggestive of smear negative and extra pulmonary tuberculosis such as:

-Smear negative Sputum and Bronchi alveolar lavage specimens.

- Painless lymphadenopathy with or without abscess formation.

-pleural aspirates from cases suspected of Tuberculous pleural effusion.

-Urine samples from cases suspected of genitourinary tuberculosis

-Discharge pus and draining fluid from cases suspected of bone and skeletal tuberculosis.

-CSF in cases suspected of tuberculous meningitis.

-other body fluids like ascitic, pericardial with suspected extra pulmonary tuberculosis involvement.

EXCLUSION CRITERIA:

Patients on Anti Tuberculosis Treatment.

STUDY PERIOD:

January 2014- SEPTEMBER 2014.

SAMPLE SIZE:

100 SAMPLES

ETHICAL CONSIDERATION:

Ethical and research clearance was obtained from the Ethical committee Stanley Medical College. Permission to conduct the study was sought from the respective hospital authorities. Informed consent was obtained from the patients before enrolment in to the study.

STATISTICAL ANALYSIS:

Statistical analysis was done with PASW (Predictive Analysis SoftWare), Statistics-16 version by statistician. P value obtained by Chi square test.

SAMPLE COLLECTION

1. The patient was given a sterile wide mouthed , leak proof container (50 ml Tarsons tubes) for sputum collection.
2. Patient was advised to cough and expectorate the sputum, and not spit the saliva.
3. If the patients was able to produce sputum then induced sputum was collected .

EXTRA PULMONARY SPECIMENS

All the patients were clearly explained about the procedure and the samples were collected after following universal precautions .

1.BODY FLUIDS: By the treating physician, under strict aseptic precautions around 10-15 ml of body fluids such as pleural, Ascitic fluid was collected by aspiration.

2. PUS SAMPLE : Under strict aseptic precautions using needle and syringe as much material as possible of pus was aspirated from the abscess. eg. Cold abscess and Psoas abscess and cutaneous abscesses.

3.BRONCHIO ALVEOLAR LAVAGE FLUID.

Bronchio alveolar lavage fluid was collected in a sterile tarson tubes around 10 -15ml from patients with smear negative and suspected pulmonary Tuberculosis patients.

All the samples were collected in duplicate that is in 2 tarson tubes one forZiehl- Neelsen , Auramine O stain &MGIT Culture and another for Gene Xpert.

TRANSPORTATION:

All the samples were transported immediately to the Microbiology laboratory, Stanley Medical College and processed as soon as possible to prevent contamination. If there is a delay in specimen processing it was stored at 4 -8° C in refrigerators. No sample was stored more than 72 hours.

SPECIMEN PROCESSING:

LABORATORY SAFETY:

Nosocomial transmission of M.tuberculosis from patients or specimens is of major concern to the health care workers because of the low infective dose of M.tuberculosis for humans (50 % infective dose , < 10 bacilli) , from the known (or) suspected cases of tuberculosis patients . This should be considered potentially infectious and so the specimens should be handled with outmost care and appropriate precautions. Aerosol production and other microbial contamination during specimen processing were controlled by using appropriate properly functioning Biosafety Cabinets classllb 2 and separate air conditioned room.

SMEAR PREPARATION:

Smear was prepared inside the Biosafety Cabinet classll b 2, for proper disinfection UV light was switched on for half an hour before and after handling the specimen inside the cabinet. Clean glass slides of 2 numbers were taken and patient details were marked. The container was opened and the specimen was taken with a sterile 4 mm Nichrome loop and it was spread it over the glass slide for about 1 x 2 cms. It was allowed to air dry and heat fixed. One slide was used for

acid fast staining by ziehl-Neelsen technique and the another slide was used for fluorescent staining (Auramine O staining).

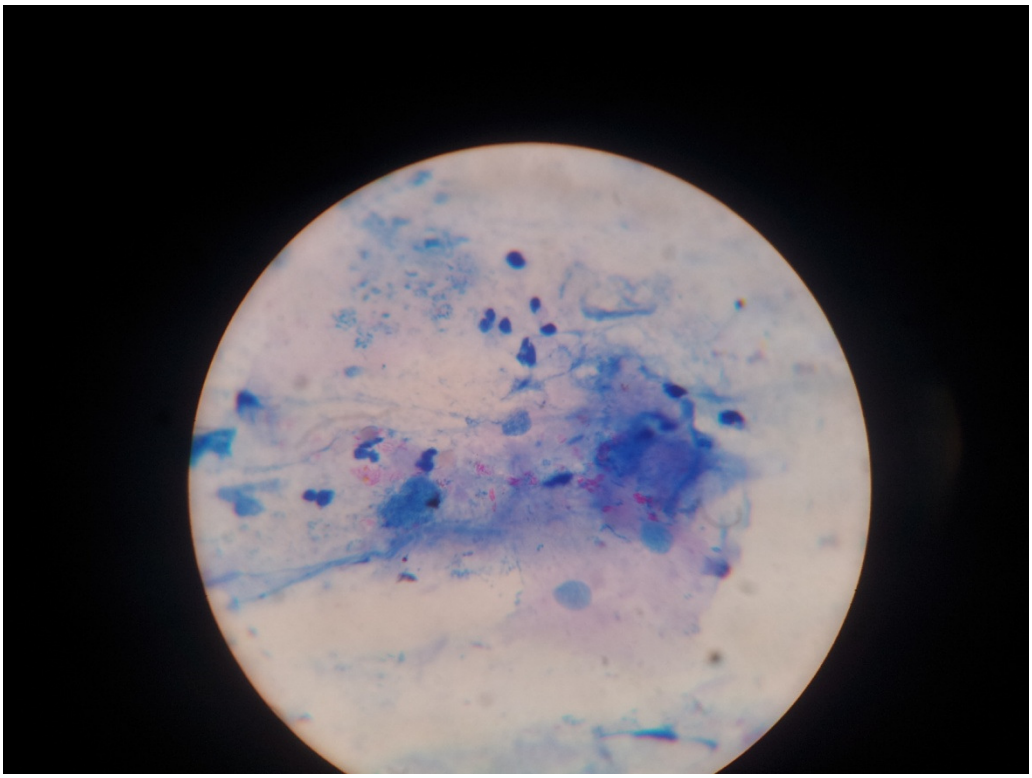
STAINING METHODS

Modified Ziehl – Neelsen staining procedure(cold method)

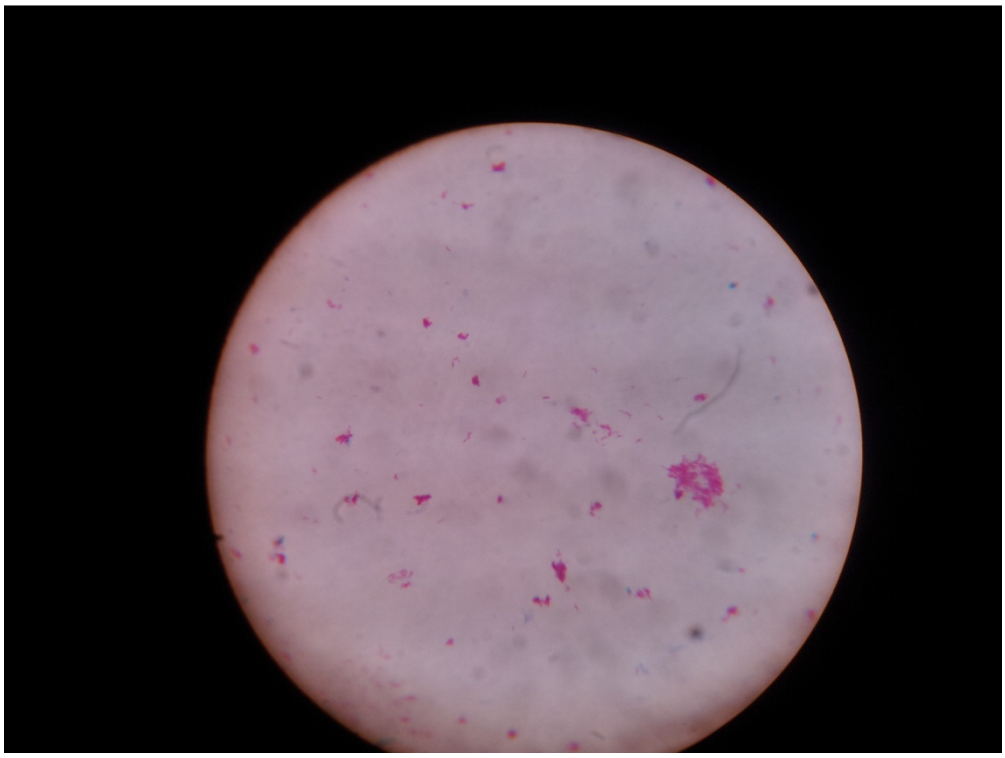
Slides were placed on the staining rack with the smear side facing upwards

1. The smear was flooded with strong Carbol – fuchsin.
2. Waited for 8 min.
3. The slide was rinsed with distilled water until all the stains were washed away.
4. Again the slide was flooded with 3% Acid Alcohol solution fordecolourisation.
5. After 3 minutes slide was thoroughly washedwith distilled water. Excess water was drained.
6. Again the smear was flooded with counter stain Methylene blue for 2 minutes
7. After 2 minutes the slide was thoroughly rinsed with distilled water and allowed it to air dr

MICROSCOPY
ZN DIRECT SMEAR(AFB POSITIVE)



MGIT CULTURE SMEAR(AFB POSITIVE)



Reporting of Smears:

The slides were examined and interpreted by the following RNTCP grading.

EXAMINATION FINDING(AFB PER OIL IMMERSION FIELD	RESULT	GRADING	N0 OF FIELDS TO BE EXAMINNED
> 10 AFB	Positive	3+	20
1 – 10 AFB	Positive	2+	50
10 – 99 AFB	Positive	1+	100
1 -9 AFB	Positive	SCANTY	100
No AFB	Negative	Negative	100

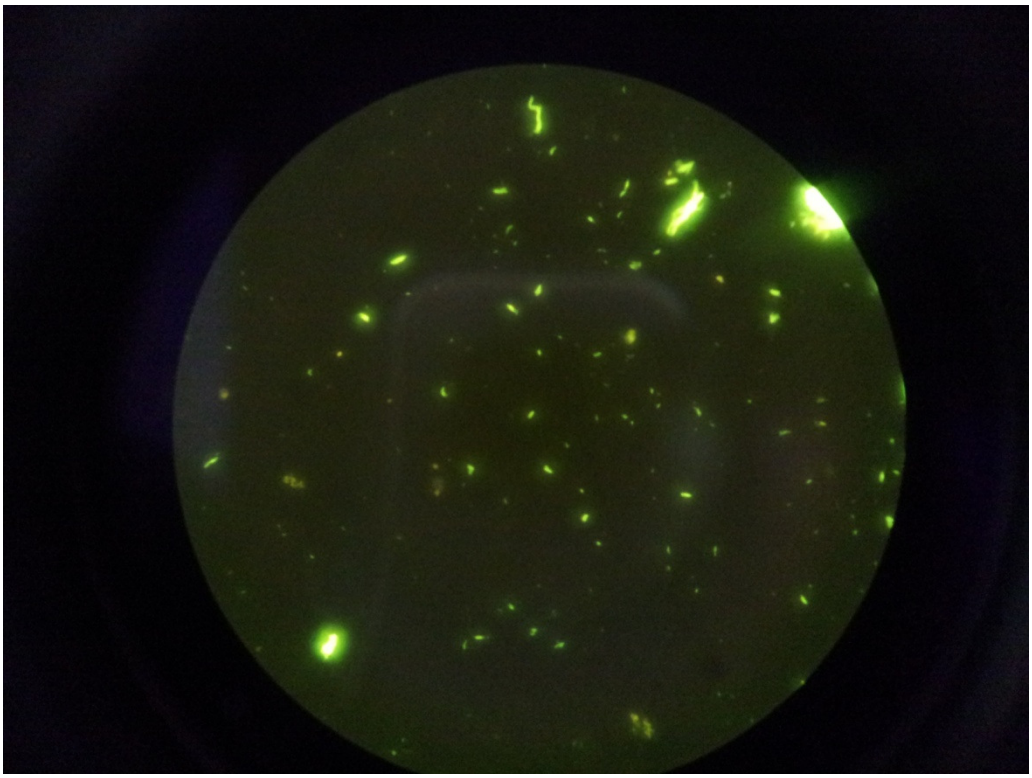
AURAMINE O STAINING:

a) Heat fixed, dried smears were covered with auramine O and allowed to stand for 20 min.

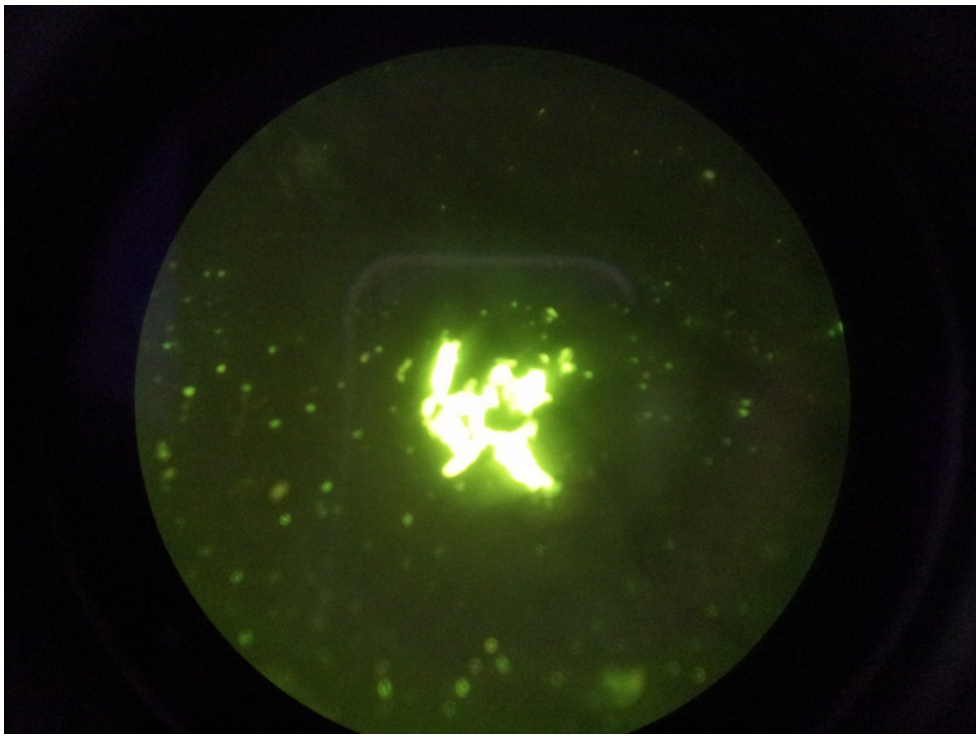
b) After 20 min smears were washed with distilled water and again decolorized with 3% acid alcohol for 3min.

c) The slide was again washed with distilled water and counterstained with potassium permanganate solution for 2 min.

FLUORESCENT MICROSCOPY
AURAMINE O DIRECT SMEAR



AURAMINE O CULTURE SMEAR(MGIT)



d) The smears were allowed to air dry and were examined with fluorescent (LED) microscopy under 40 X for screening with blue light.

e) In positive slides smears AFB bacillis were seen as bright yellow or green rods under dark background.

CULTURE METHOD: -

Tubercle bacilli was cultured in liquid culture medium MGIT (Mycobacterial Growth Indicator Tube). Since liquid culture medium is more prone for contamination, Digestion, Decontamination and Concentration technique is essential for specimens, especially if it known to be contaminated with commensal organisms such as sputum.

Hence this study includes smear negative sputum samples and extra pulmonary samples. Specimens processed accordingly by the following described methods given in MGIT manual provided by Becton Dickinson.

For Sputum Samples - NaOH – NALC decontamination, digestion procedure was followed and this is the standard recommended procedure to be used with MGIT, which is also recommended by CDC.

Materials and Methods:

Materials Required

- Disposable 15 ml plastic tubes (Tarson tubes)
- Sterile NaOH – NALC – Sodium Citrate solution
- Phosphate buffer Ph 6.8 (0.067m)
- Centrifuge with minimum 3000 – 3500 rpm
- Vortex mixer , shaker
- Pipettes / Tips.

Preparation of NaOH – NALC – sodium citrate solution

a) 4% NaOH – Kills the normal flora.

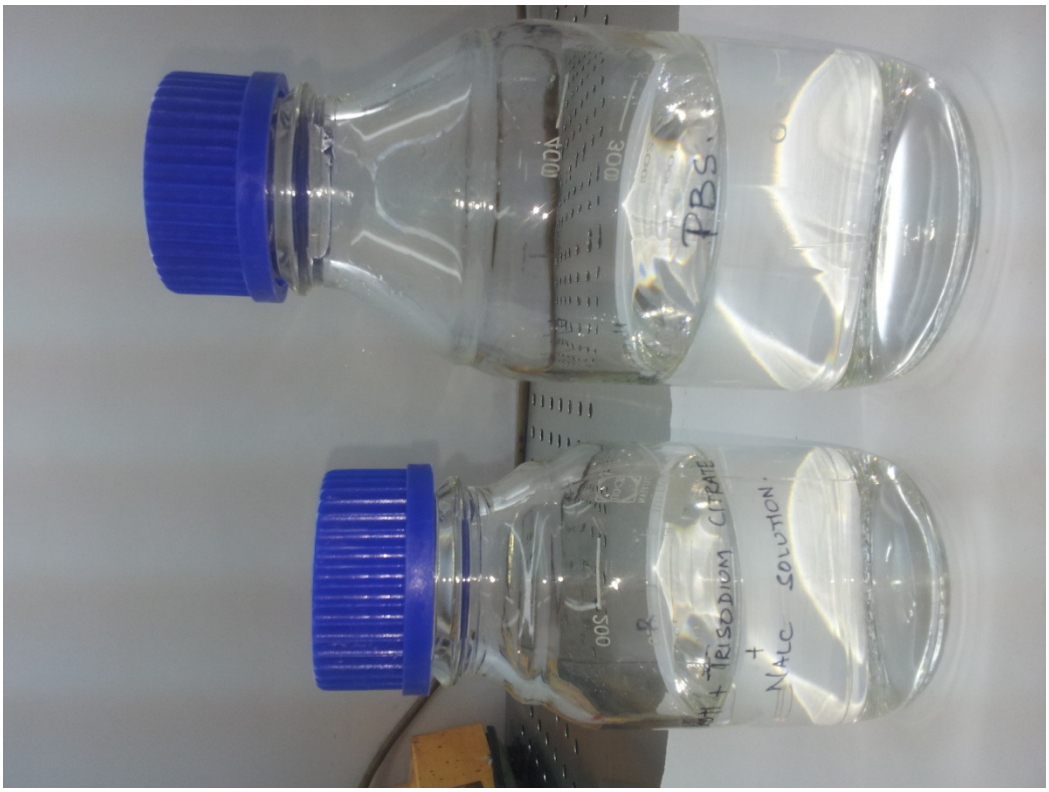
b) 9% Citrate – binds the heavy metal ions that might be present in the specimen that could inactivate the N – acetyl – L – cysteine (NALC).

c) NALC – Mucolytic agent which liquefies and release AFB from the organic matrix of specimen.

1. 20 g of NaOH was added to 0.5 l D.W (4 % NaOH).
2. 14.5 g Na citrate was added to 0.5 l D.W (2.9 % Na Citrate).
3. 2 solutions were mixed in a 1 liter bottle and autoclaved for 20 minutes @ 121°c at 20 Psi.

MGIT (PANTA, OADC)

NAOH NALC , PBS



Preparation of Phosphate buffer 0.067 m Phosphate buffer, PH 6.8

This contains 2 stock solutions:

a) Disodium Phosphate (Solution A) – Prepared by dissolving 9.47 d of anhydrous Na_2HPO_4 in l lit of D.W.

b) Mono Potassium Phosphate (Solution B) – Prepared by dissolving 9.07 g of KH_2PO_4.

After preparing their stock solutions A & B both were mixed together and PH was adjusted to 6.8 + 0.2 using PH strips. Finally the solution was transferred in to small agent bottles for daily usage and autoclaved at 121°c for 20 minutes at 21 Psi and was stored at 2.8°c.

Sputum Samples:

a) Around 2 – 5 ml of sputum was collected in 15 ml Tarson tube.

b) Equal amount of NaOH – NALC – tri solution citrate solution was added to the specimen and the cap was closed tightly.

c) Falcon tubes were vortexed lightly for about 15 – 30 seconds and the tubes were inverted so that the whole specimen is exposed to NaOH – NALC solution.

d) Waited for 15 – 20 minutes after adding NaOH – NALC solution andvortexed lightly every 5 – 10 min.

e) If the specimen was still mucoid, not liquefied properly even after 15 min, about (30 – 35 mg/ms) of NALC powder was added directly to the specimen tube and mixed well.

f) At the end of 15 – 20 minutes, the Phosphate buffer (PH 6.8) was added up to the top ring on the centrifuge tube. Then the tube was mixed well by inverting it several times.

g) Then centrifuged the tube at a speed of 3000 rpm for 15 – 20 min.

h) After centrifugation the tubes were allowed to stand for 5 minutes to allow the aerosols to settle down. Then the supernatant was carefully decant in to the container containing 5 % Lysol (Mycobacterial disinfectant).

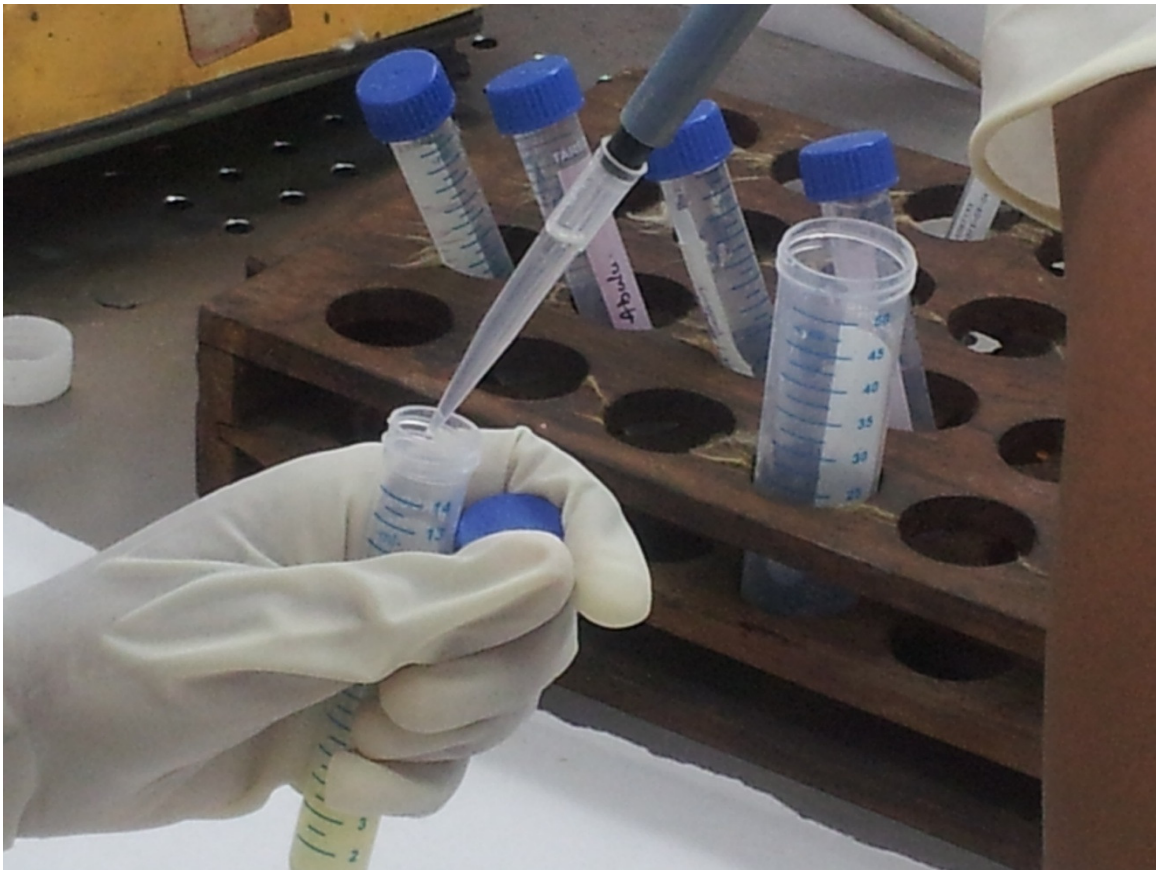
i) Finally to the sediment 1 ml of Phosphate buffer (PH 6.8) was added to re suspend the sediment with the help of pipette.

j) The resuspended pellet was used again for inoculation in to MGIT (Mycobacterial Growth Indicator Tubes) and for making smears.

EXTRA PULMONARY SPECIMENS.

A) 1. **STERILE BODY FLUIDS** such as pleural fluid, Ascetic fluid were collected aseptically.

PROCESSING



VORTEXING

2. If the specimen was 2 – 3 ml, it was decontaminated as of sputum

3. If the specimen volume was more than 10 ml, then it was concentrated by centrifugation at about 3000 rpm for 15 min.

4. After centrifugation it was decontaminated with equal amount of NaOH - NALC solution, after discarding the supernatant.

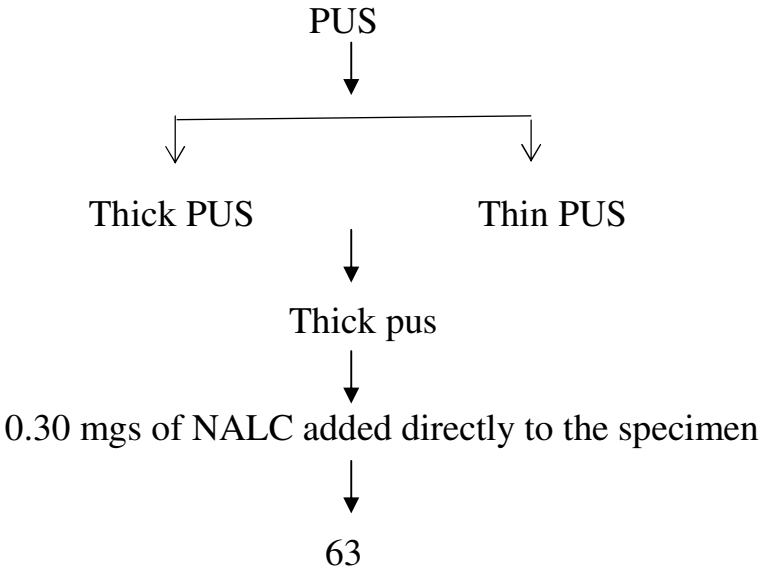
5. Phosphate buffer solution (PH 6.8) was added up to the top ring and again centrifuged for 15 – 20 min.

6. Supernatant was discarded in mycobacterial disinfectant and the pellet was resuspended in 1 ml of phosphate buffer (PH 6.8)

7. Around 0.5ml of processed specimen was inoculated in to MGIT tube using pipette.

B) PUS Sample

1. Digestion Decontamination procedure varies for Thick and Thin PUS



Wait for 15 min

After liquefying equal amount of NaOH – trisodium citrate – NALC solution was added for decontamination

↓

Then PBS was added until the rim of the tarson tube

↓

Centrifuged for 15 min @ 3500 rpm

↓

Supernatant was discarded

↓

Pellet was resuspended in 1 ml of PBS

↓

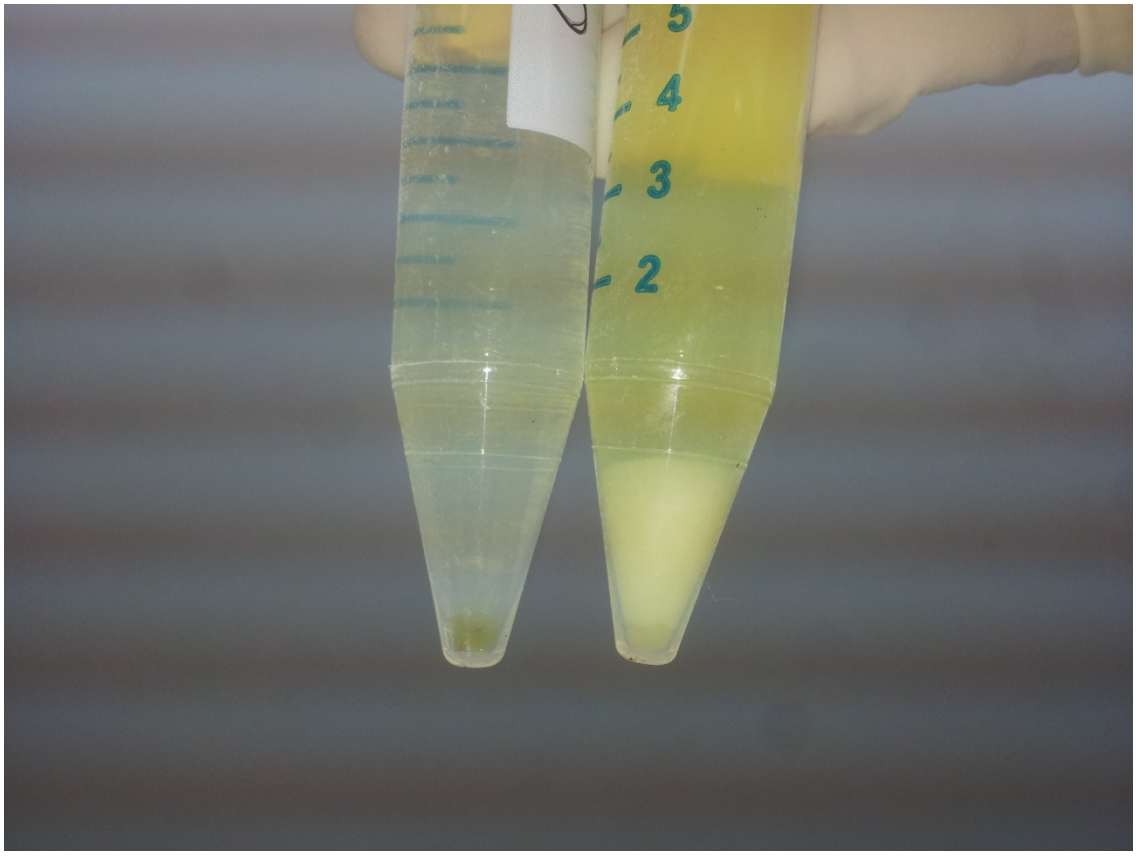
Final resuspended specimen was used for inoculation in to MGIT tubes

Thin PUS

↓

< 10 ml	>10ml
↓	↓
Processed as of sputum with NaOH – NALC solution	Centrifuged first for 15 mi @ 3500 ↓ Supernatant discarded ad pellet was again

SEDIMENT AFTER PROCESSING



resuspended in PBS solution

Again processed as of sputum with NaOH – NALC solution.

SWABS

SWABS

↓

Swabs were immediately transferred in to sterile tarson tubes

↓

Around 2 ml of sterile distilled water was added

↓

Around 2 ml of NaOH – NALC solution was added

↓

Centrifuge tubes were tightly closed with the swab and vortexed for 20 – 30 seconds

↓

Allowed it to stand for 15 min.

↓

Swabs were removed with the help of forceps after squeezing the liquid out of swab

↓

PBS solution was added until the top ring

↓

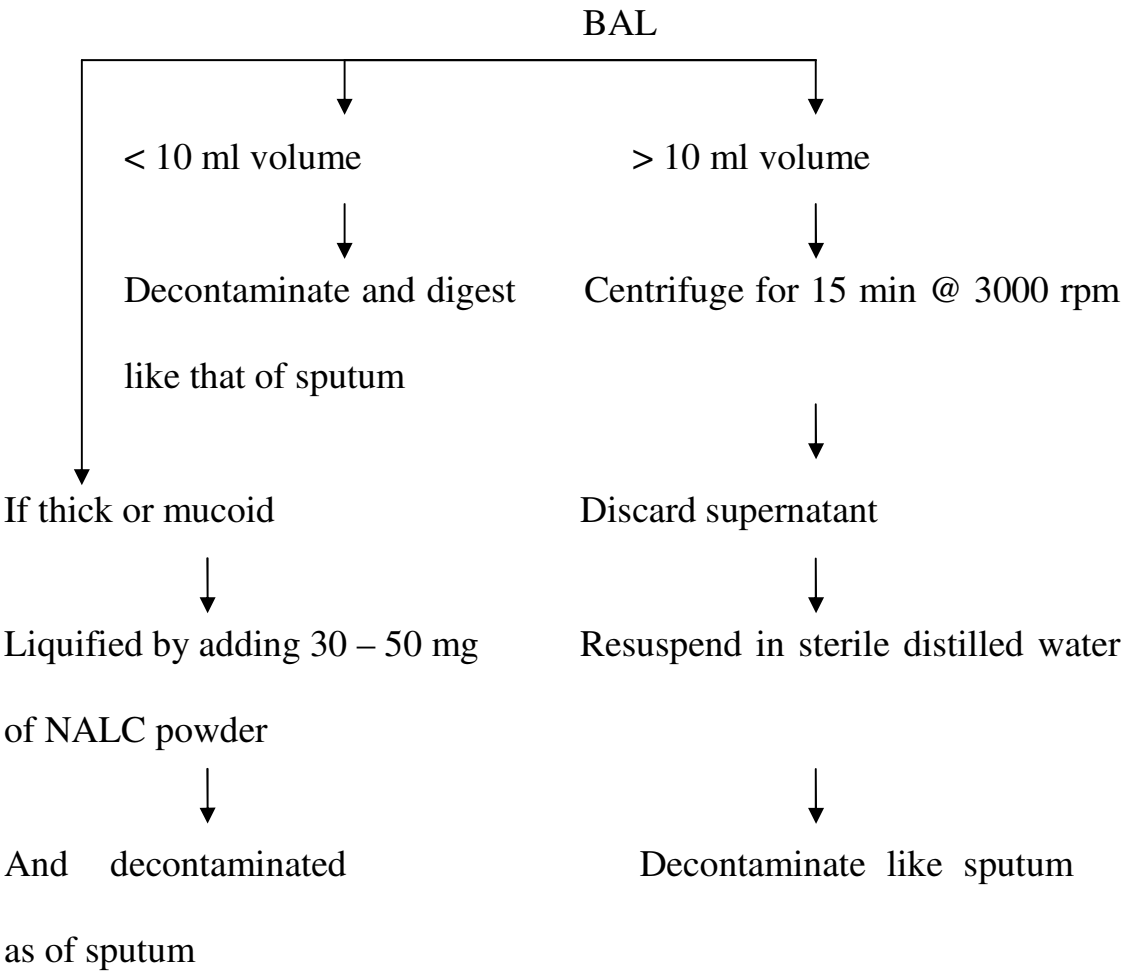
Centrifuged @ 3000 rpm for 15 min

Supernatant discarded, sediment was resuspended with 1 ml PBS

↓

Final resuspended solution was used for smear preparation and inoculation in to MGIT tubes.

BAL was treated as sputum



PREPARATION AND INOCULATION FOR CULTURE

1. Reagents

A) MGIT Medium

- MGIT 960 tube contains 7.0 ml modified 7H9 broth base (1000 ml of purified water contains)
  - Modified Middlebrooks 7H9 broth base 5.9 gm
  - Casein Peptone 1.25 g
- Fluorescent sensor embedded in silicone on the bottom of the tube.
- Tube contains 10 % CO2 and it was capped with polypropylene screw caps.

B) MGIT growth Supplement (enrichment)

For manual MGIT (OADC, 15 ml) is used as growth supplement which contains 15 ml of the following

- Bovine Albumin - 50 .0 gm
- Dextrose - 20.0 gm
- Catalase - 0.03 gm
- Oleic acid - 0.1 gm

- Polyoxyethyline state (POES) - 1.1 gm

C.MGIT PANTA

Since liquid medium is more prone for contamination, this can be reduced by supplementing the medium with mixture of antimicrobial PANTA prior to the inoculation of specimen.

Each vial of MGIT PANTA contains a lyophilized mixture of the antimicrobials with the concentrations given below

- Polymyxin B – 6000 units
- Amphotericin B – 600 µg
- Nalidixic acid – 2400 µg
- Trimethoprim – 600 µg
- Azlocillin – 600 µg

2. PROCEDURES

a) Reconstituting PANTA

- Reconstituted MGIT PANTA with 15.0ml of MGIT growth supplement and mixed until completely dissolved.
- The enrichment reconstituted PANTA was added to the MGIT medium prior to inoculation of specimen.

b) Inoculation into MGIT medium:

- MGIT tubes were labelled with specimen name, number and date of inoculation.
- 0.8 ml of MGIT growth supplement / PANTA was added aseptically in to each tubes.
- Using a sterile pipette tips, 0.5 ml of well mixed processed / concentrated specimen was aseptically added to the MGIT tubes.
- Separate pipette tips were used for every specimens.
- The inoculated tubes were recapped tightly and mixed by inverting the tubes several times
- The inoculated tubes and caps were wiped with 5 % Lysol and left at room temperature for 30 minutes.
- All the works were done under Biosafety cabinet class II B2.

d) Precautions taken care during Processing:

- All the additions were done inside in a biosafety cabinet.
- Tubes were not opened several times.

- MGIT tubes were opened as short a period of time as possible.
- Tubes were recapped tightly.
- Volume greater than 0.5 ml of decontaminated specimen may disturb the PH of the medium, and leads to contamination and so it was maintained strictly.

e) INCUBATION:

- All inoculated tubes were incubated in 37°c ± 1°c incubator
- The caps were tightly closed and it was not shaked during incubation. This helped in maintaining the oxygen gradient in the medium.
- The tubes were incubated until it became positive. After six weeks of incubation reported as negative.

QUALITY CONTROL OF AFB SMEAR STAINING AND TESTING OF MGIT MEDIUM:

For positive control M.tuberculosis ,H37Rv(ATCC 27294) was obtained from NATIONAL TUBERCULOSIS RESEARCH CENTRE ,CHETPET.

The same was used for positive control for ZN & AO staining ,and for negative control gram negative organisms was used.

One MGIT tube was inoculated with H37Rv for & this was used everyday, as positive control while reading the tubes.

An uninoculated MGIT tube was used as a negative control.

Few random samples were also sent to the Bacteriology department, NATIONAL TUBERCULOSIS RESEARCH CENTRE ,CHETPET for correlation of results from both the centres. Both the centres showed identical results.

f) DETECTION OF POSITIVE GROWTH:

- Starting from the second day onwards, tubes were screened for growth by both visually and Micro MGIT instrument.
- Mycobacterial growth appeared granular, with grain of flakes appearance and not very turbid, but the contaminated bacterial growth appeared very turbid.
- Growth especially of M tuberculosis Complex settles at the bottom of the tube.

MGIT POSITIVE

MGIT NEGATIVE

Mycobacterial Growth will utilise all the O_2 and so the quenched fluorescent substance will be released and it will fluoresce.

- MICRO MGIT instrument will detect the flourescene and will show the positivity.
- Calibrator control should be checked every day before taking up the reading.

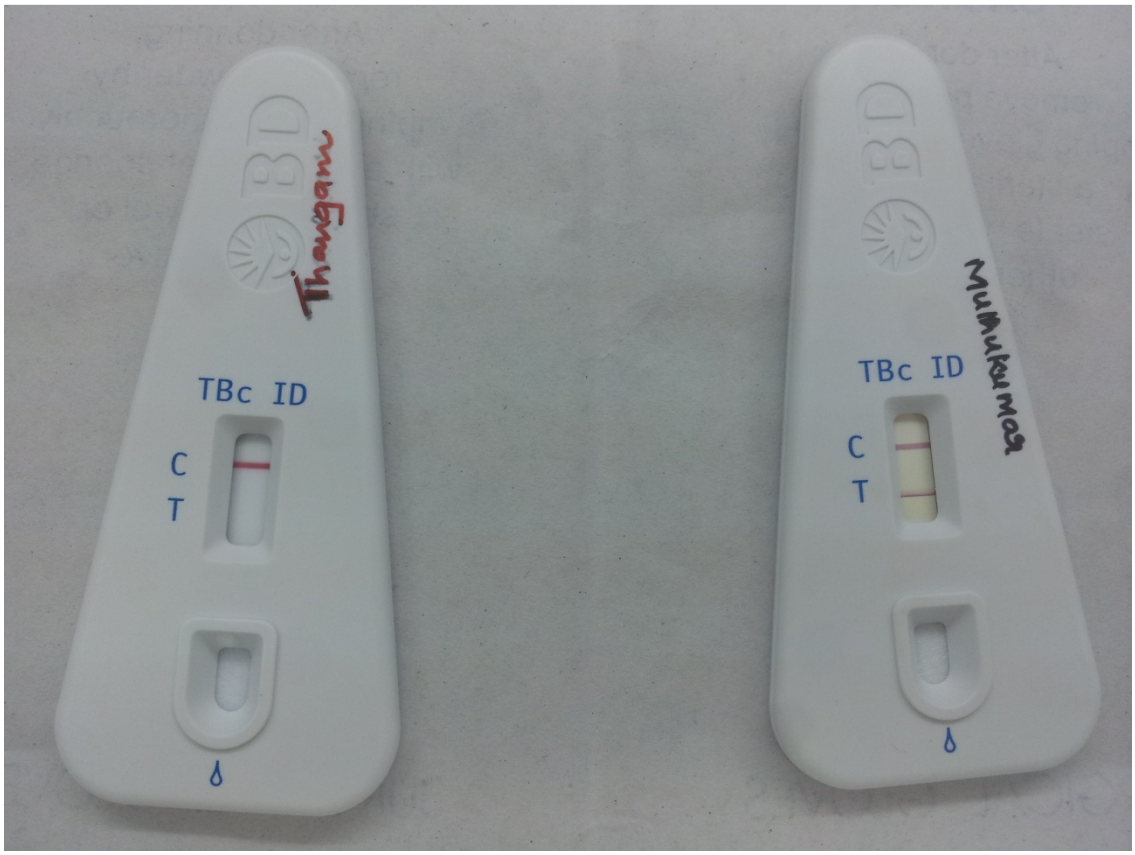
G. WORK UP OF POSITIVE CULTURES:

- Using a sterile pipette tips under sterile aseptic precautions , Smear was prepared from the positive tube , heat fixed and left over night and stained with ZN staining.
- And the smear was examined under oil immersion for the presence of AFB.
- After confirming Acid fast Bacilli in the Positive tube.

Rapid card test(Immunochromatographic test) to differentiate between Mycobacterium other than tuberculosis was done.

MGIT POSITIVE

TBC ID NEGATIVE & POSITIVE



TBCID is a rapid chromatrographic immunoassay

This qualitative test detects the Mycobacterium tuberculosis complex (MTbc) antigen from AFB smear – positive MGIT tubes.

Mycobacterium tuberculosis complex includes M. tuberculosis complex includes M.tuberculosis, M.bovis, M.africanum, M.microti.

PRINCIPLE:

- This detects MPT64, a mycobacterial protein fraction that is secreted from MTbc cells during culture.
- 100 µg of sample from the positive tubes was added to the well.
- MPT64 antigen , if present binds to anti MPT64 antibodies conjugated to visualizing particles on the test strip.
- The antigen – conjugate complex migrates across the test strip to the reaction area and is captured by a second specific MPT 64 antibody applied to the membrane.

- Positives were seen as a colored reactions, produced by the labelled colloidal gold particles and was visualized as a pink to red link.

PROCEDURE

- All smear positive MGIT tubes were tested in the TBCID device within 10 days after positivity.
- The refrigerated devices were brought to ambient room temperature prior to testing.
- TBCID device was labelled and it was kept on a flat surface inside the Biosafety cabinet.
- The sample was thoroughly mixed by inverting the tube several times.
- 100 ml of sample was added using a sterile tips.
- Result was read after 15 min

INTERPRETATION OF RESULTS

A) POSITIVE TEST FOR TBC (MPT 64 antigen present)

- Pink to red line appears at the test 'T' position and the control ''c'' position in the read window.

B) NEGATIVE TEST FOR TBC (No MPT 64 antigen detected)

- No pink to red line was visible at the test ‘‘T’’ position of the read window.

C) INVALID TEST

- The test was invalid if no pink to red line was visible at the control ‘‘C’’ position in the read windows.

REPORTING OF RESULTS

1) Positive test was reported as MTb complex.

Negative test was reported as AFB non –MTbc.

DRUG SUSCEPTIBILITY TESTING

Before doing Antituberculous sensitivity all the positive tubes were subcultured in to BAP or CAP and SDA to rule out contamination with otherbacterias and fungi.

PRINCIPLES OF THE TEST

- TB cultures were subjected to growth in the presence of a known concentration of a test drug. A control was also included with no addition of drug. If the isolate grows in control tube but not in presence of drug was considered susceptible. On the other hand if it grows in both tubes it was considered resistant.

- Proportion method was used for the susceptibility testing. In this method the resistance was established at the 1 % level for most of the drugs. This means that if 1 % or more of the total test bacterial population is resistant to a drug it is considered as resistant for clinical purposes.
- The bacterial inoculum in the control was hundred fold less than the inoculum in the drug containing medium

REAGENTS

BACTEC MGIT 960 SIRE KIT for critical concentrations contains the following drugs in lyophilized form

a) DRUGS

- Streptomycin – approximate lyophilized drug per vial – 332µg
- Isoniazide – approximate lyophilized drug per vial – 33.2µg
- Rifampin – approximate lyophilized drug per vial – 332µg
- Ethambutol – approximate lyophilized drug per vial – 1660µg

3) SIRE(Streptomycin, Isoniazide, Rifampicin, Ethambutol)

SUPPLEMENT

> Bovine albumin - 50.0g

> Dextrose - 20.0 g

> Catalase - 0.03g

> Oleic acid - 0.6 g

STORAGE

Upon receipt, the lyophilized drugs were stored at 2-8°c. It was reconstituted prior to use once opened and reconstituted, the left over drug solutions were aliquoted and it was frozen at - 20°c. Once thawed the leftover drugs were discarded which was not restored or refreezed.

PROCEDURES

4) a) RECONSTITUTION OF LYOPHILIZED DRUGS

Each lyophilized drug vial was reconstituted with 4 ml of sterile distilled / deionized water, which was mixed thoroughly and make sured that the drug was completely dissolved.

b) Addition of a drug to a medium

100 µl (0.1ml) of reconstituted drug solution was added aseptically, inside the BSC, in to the labelled MGIT tubes. This resulted in the following

Streptomycin - 1.0µg/ml

Isoniazide - 0.1 µg/ml

Rifampin -1.0µg/ml

Ethambutol -5.0µg/ml

AST was done within fivedays of positivity.

For 1st and 2nd day

- Tubes was vortexed and allowed to stand for 5 min, then the supernatant was used to inoculate into the drug containing tubes, & 1 in 100 dilution of the tube was inoculated in to the control tube.

Similarly for Day 3, 4&5

- 1 ml of culture suspension was mixed with 4 ml normal saline to form 1 in 5 dilution.
- From this 100 µl was taken and added in to 10 ml of normal saline to make in to 100 dilutions.
- 800 µl of SIRE supplement was added on all the 5 MGIT tubes.

- 500 µl of 1 in 100 dilution of culture suspension was added in 1st tube which serves as a growth control tube.
- 500 µl of 1 in 5 dilution of culture suspension was added in rest of the tubes containing Streptomycin, Isoniazide, Rifampicin, Etambutol antibiotics respectively.
- Then the tubes were incubated @ 37°c and reading was started form second day onwars for the the growth.

RESULTS: -

- On the day the control tube showed positivity, rest of the antibiotic containing tubes were read and results interpreted.

REPORTS: -

- If there was no growth on the drug containing tube it was susceptible.
- If there was growth present on the drug containing tube it was resistant.

Gene Xpert

All the samples were taken to National Tuberculosis Reseach Centre, chetpet for Gene XpertTB/RIF analysis. It is a single step procedure Semi nested multiplex real time PCR.

1. Clinical specimens which were collected in Tarsonstubes were treated with a sodium hydroxide and isopropanol-containing sample reagent/lysis buffer (SR), in the ratio of 4:1 and kept at room temperature for 15 min and were also vortexed twice, this step reduces the viability of *M. tuberculosis* in specimen. After which around 2 ml was added to the sterile cartridges manually.Each specimen loaded cartridge has a barcode, which when scanned will indicate the position of its placement.

2.Subsequent processing is fully automated.

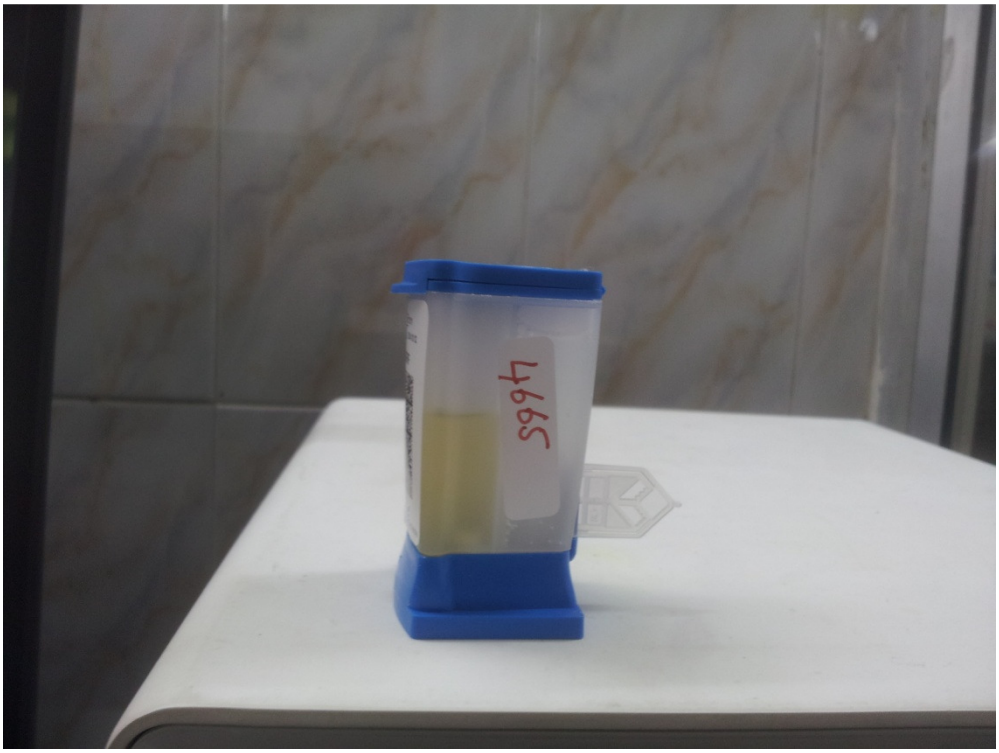
3.This assay employs single-use 11 chambered plastic cartridges that are preloaded with liquid buffers and lyophilized dried beads with necessary reagents for sample processing, DNA extraction and seminested real time-PCR.

4..The cartridge includes a syringe drive, a rotary drive and a filter at the base upon which M. tuberculosis bacilli gets deposited after liberated from the specimen.

5. The platform has a sonic horn that inserts into the cartridge base to cause ultrasonic lysis of the bacilli which releases the DNA , is then amplifies 192 bp segment of the rpoB gene by a hemi-nested real time-PCR reaction .

GENEXPERT

CATRIDGE



6. QUALITY CONTROLS

The also has lyophilized Bacillus globigii spores as an internal sample processing and PCR control. The B. globigii PCR assay is multiplexed with the M. tuberculosis assay.

Mycobacterium tuberculosis is detected by the five overlapping molecular probes (probes a,b,c,d.e) that are complementary to the 81 bprpoB core region . M. tuberculosis is detected ,at least two of the five probes give positive signals within the cycle threshold (CT) of less than or equal to 38 cycles.

RESULTS

The B. globigii internal control is positive when the single B. globigii -specific probe produces graph at CT of ≤38 cycles. The standard user interface indicates the presence or absence of M. tuberculosis and the presence or absence of rifampicin resistance.

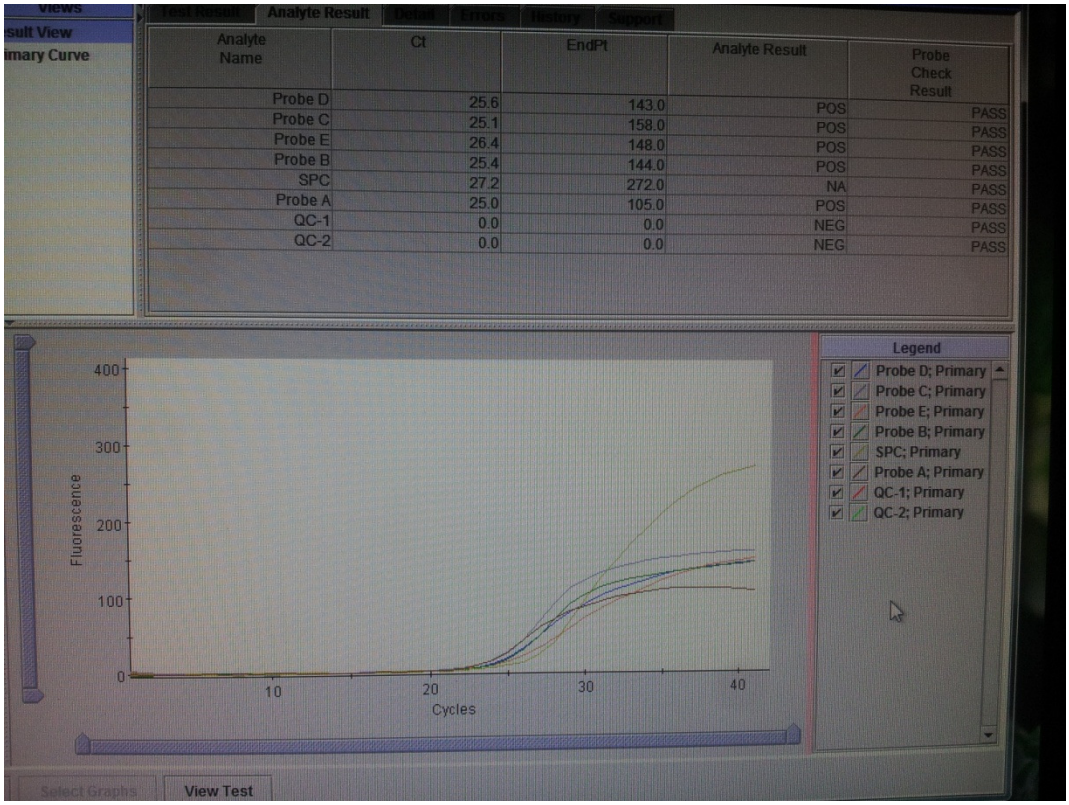
INVALID RESULTS

No graph for both M. tuberculosis and for the B. globigii internal control are reported as invalid assays.

RIFAMPICIN RESISTANCE RESULTS

SCANNING THE BARCODE

MTB GRAPHS – GENE XPERT



SEDIMENT AFTER PROCESSING

Rifampicin resistance is detected by the difference between the first (early Cycle Threshold) and last (late CT) of M. tuberculosis -specific beacon . The system was configured such that resistance was reported when ΔCT was >3.5 cycles and sensitive if ≤3.5 cycles. Since the assay terminates after 38 cycles, the assay was deemed indeterminate for rifampicin resistance if the first probe CT is >34.5 cycles and the last probe has a CT of >38 cycles .

OBSERVATION & RESULTS

Totally Hundred patients suspected of having tuberculosis infection were included in this study from the department of TB and Chest Medicine for the detection of mycobacterium tuberculosis in Smear negative pulmonary specimens and Extra pulmonary specimens .Among them sputum(40), Bronchio-alveolar lavage fluid(9) , pleural fluid(31) ,ascitic fluid(5), pus/empyema(7), Psoas abscesses(2), Breast abscess with sinus tract discharge(1), lymph node aspirates(3) were collected and processed to detect the presence of mycobacterium tuberculosis by LED fluorescent Microscopy, MGIT (Mycobacterial Growth Indicator Tube) and Gene Xpertat the Department of Microbiology periodically between January 2014 to September 2014. The results were analyzed using SPSS Software version 16.

Table-1 : AGE DISTRIBUTION:

Age in years	Pulmonary symptoms	Extra pulmonary	Total
≤ 20	4	5	9
21 - 40	9	22	31
41 - 60	21	18	39
> 60	15	6	21
Total	49	51	100
p valve	9.614		
Mean Age	56.3		
df value	3		
Asymp.Sig	0.022		

The commonest age group was 41 to 60 years.

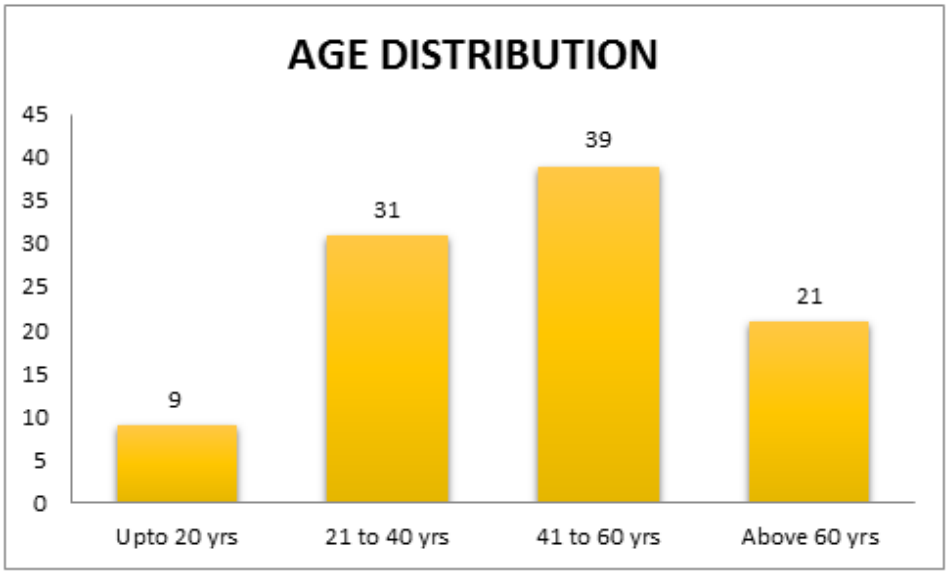
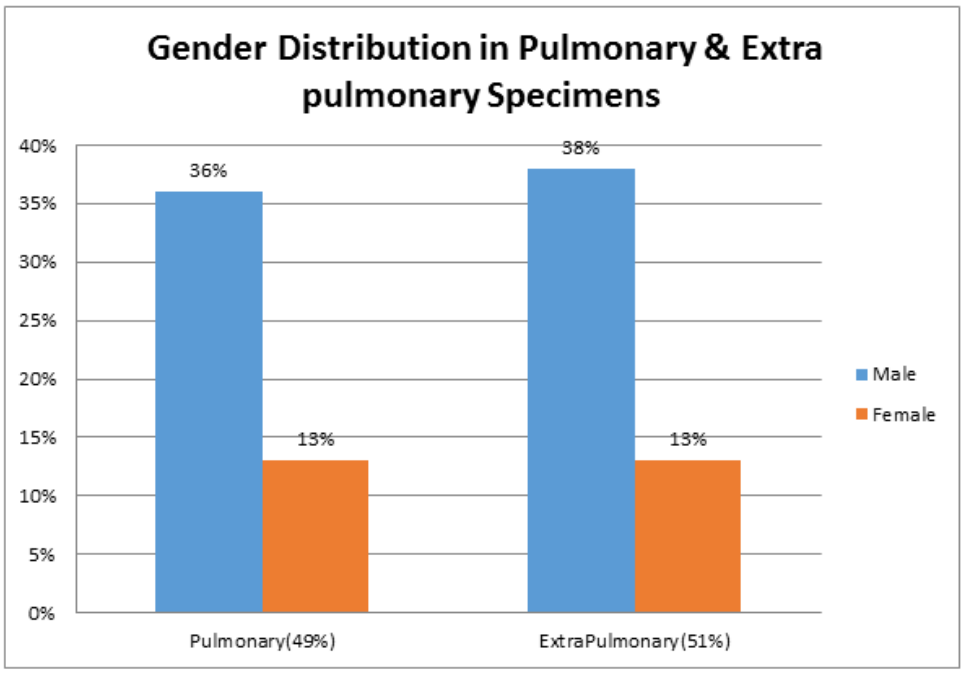

AGE DISTRIBUTION
45
40
35
30
25
20
15
10
5
0
9
31
39
21
Upto 20 yrs
21 to 40 yrs
41 to 60 yrs
Above 60 yrs

Table 2 : GENDER DISTRIBUTION IN PULMONARY AND EXTRA PULMONARY SPECIMENS.

	Pulmonary	Extra pulmonary	Total
Male	36	38	74
Female	13	13	26
Total	49	51	100
P value	0.014		
Df	1		
Fishers Exact Test	1.000		

Among tuberculosis suspected patients 74% were males, and 26% were females, this is statistically significant, and they were equally distributed in both pulmonary and extra pulmonary category.


Gender Distribution in Pulmonary & Extra pulmonary Specimens
40%
35%
30%
25%
20%
15%
10%
5%
0%
36%
13%
38%
13%
Pulmonary(49%)
ExtraPulmonary(51%)
Male
Female

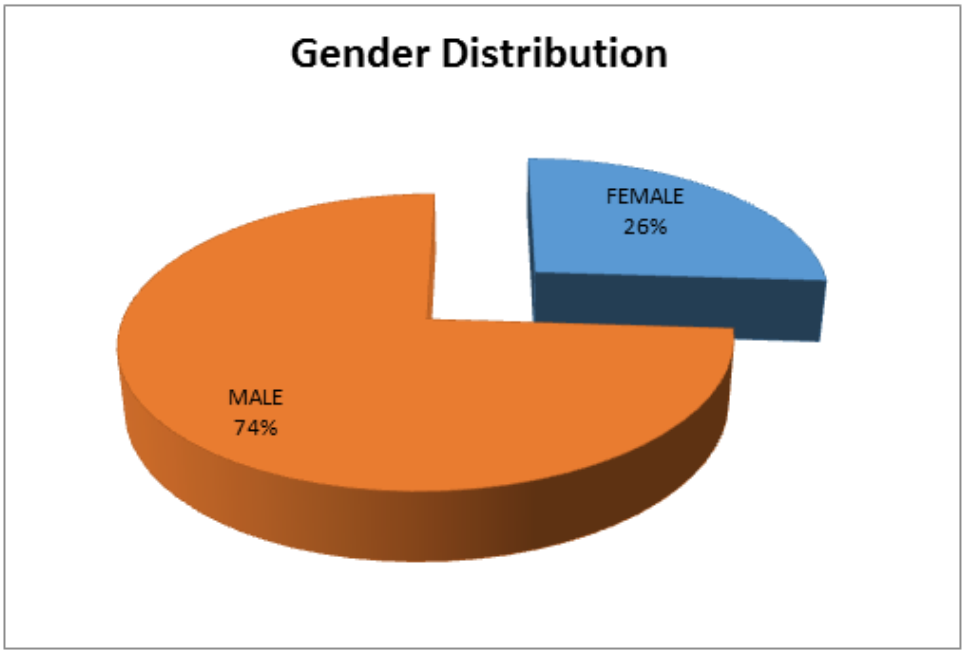

Gender Distribution
FEMALE
26%
MALE
74%

Table 3 : SOCIO ECONOMIC STATUS ACCORDING TO KUPPUSAMY CRITERIA.

Patients With Symptoms Of	Pulmonary (49)	Extra pulmonary 51	Total
Lower	-	-	-
Upper lower	1	-	1
Lower middle	25	32	57
Upper middle	22	18	40
Upper	1	-	1
P value	3.465		
Df value	3		

Among them, most of the patients belongs to lower middle class followed by upper middle class.

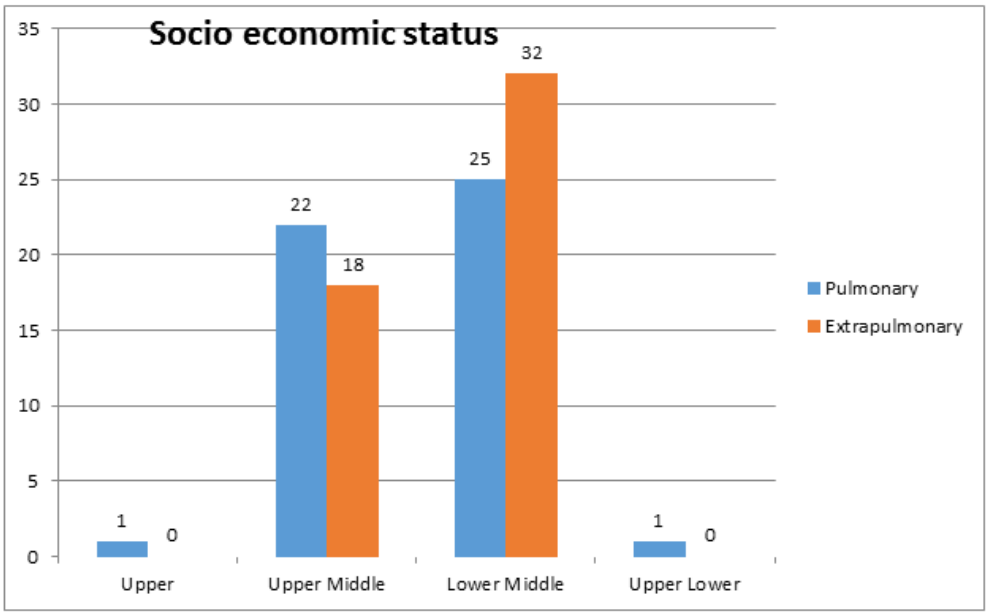


Table 4 : PRESENTING COMPLAINTS.

Complaints	**Pulmonary TB**		**Extra Pulmonary TB**		**Total**
	Male	**Female**	**Male**	**Female**	
H/O Constitutional symptom(62)	17	5	33	7	62
Cough with expectoration(22)	13	4	3	2	22
Hemoptysis(25)	22	3	-	-	25
Breathlessness, Chest Pain(40)	-	-	36	4	40
Abdominal Distention/ Ascitis(5)	-	-	3	2	5
Neck Swelling(5)	-	-	5	-	5
Breast Abscess with chest wall Discharge(1)	-	-	-	1	1

The commonest complaint was constitutional symptoms 62%, followed by breathlessness in extra pulmonary& Hemoptysis in

pulmonary TB suspected patients.

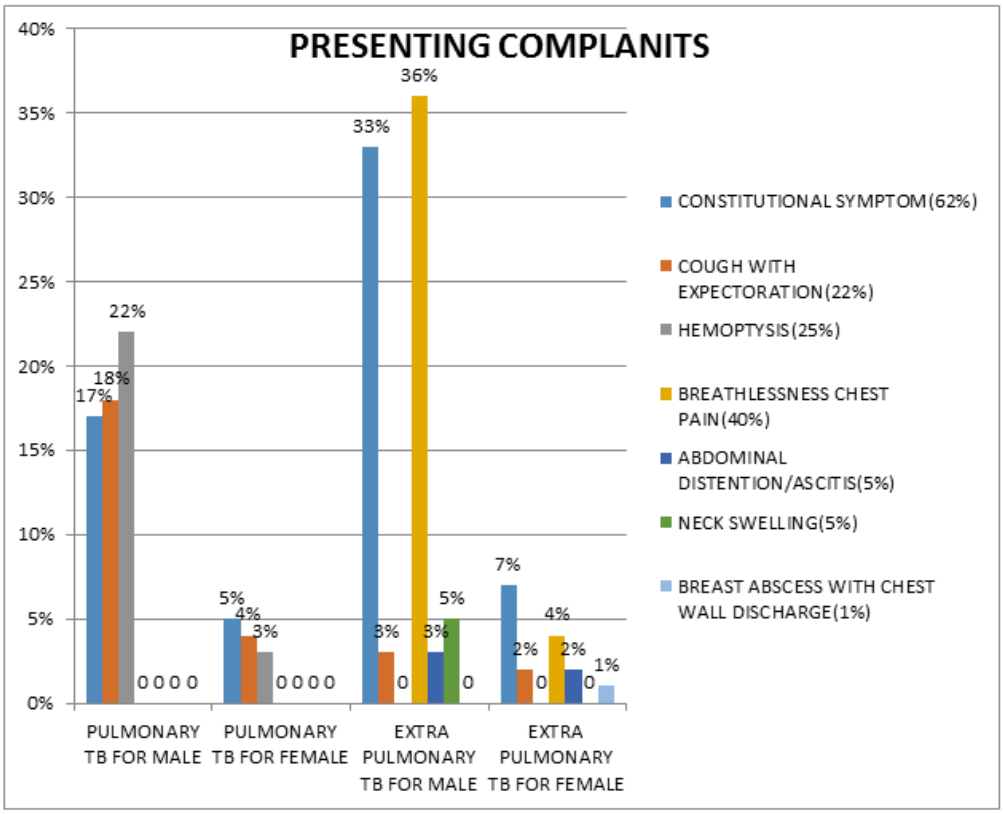


Table 5 : ASSOCIATED DISEASES.

Patients With Symptoms Of		Pulmonary	Extra Pulmonary	Total
Diabetes Mellitus(7)		4	3	7
Hypertension(2)		2	-	2
Carcinomas	Pancreatic(2)	-	2	2
	Bronchogenic(1)	-	1	1
	Laryngeal(1)	-	1	1
COPD(2)		2	-	2
Asthma/Pneumonia(2)		2	-	2
Carcinoid Lung(1)		1	-	1
DM/HT(3)		2	1	2

Most common associated diseases among the tuberculosis suspected patients was Diabetes Mellitus(7%) followed by carcinomas(4%)

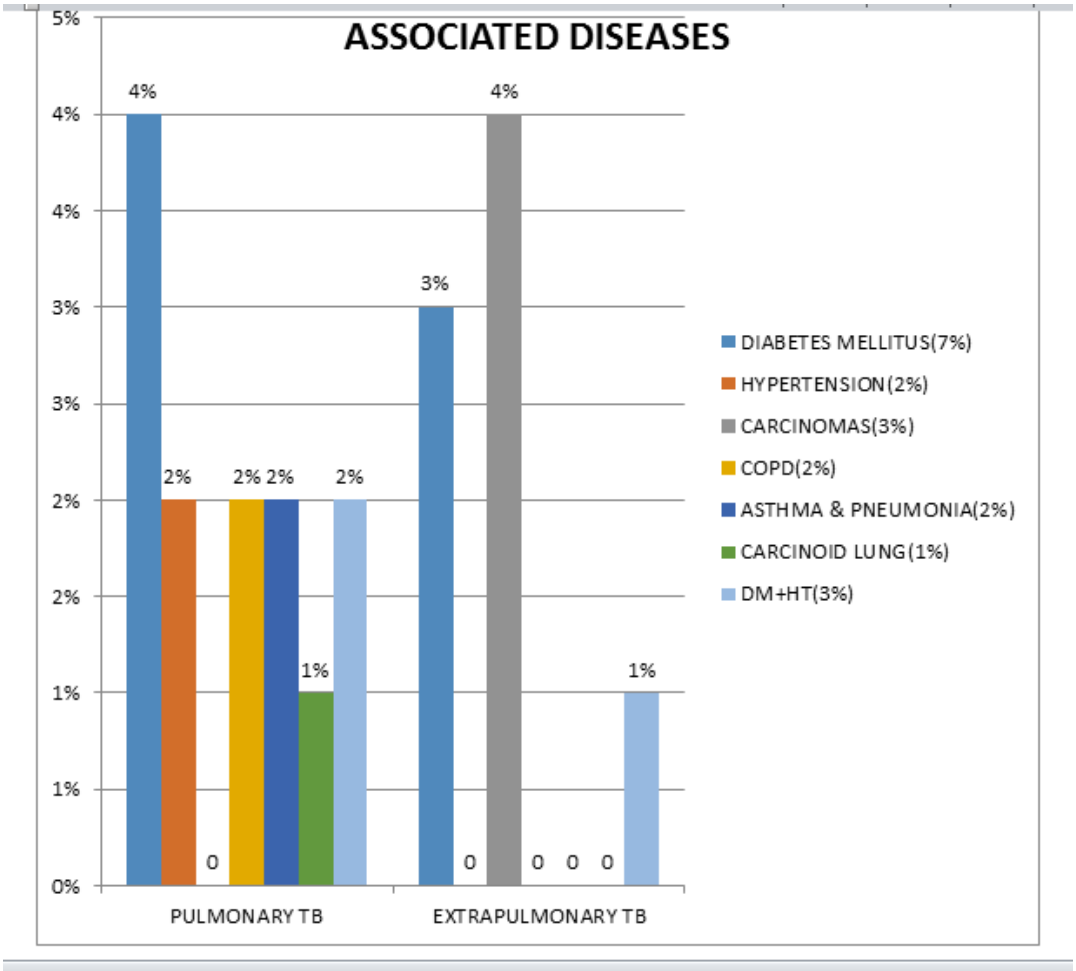

ASSOCIATED DISEASES
5%
4%
4%
3%
3%
2%
2%
1%
1%
0%
4%
2%
0
2%
2%
1%
2%
3%
0
4%
0
0
0
1%
PULMONARY TB
EXTRAPULMONARY TB
DIABETES MELLITUS(7%)
HYPERTENSION(2%)
CARCINOMAS(3%)
COPD(2%)
ASTHMA & PNEUMONIA(2%)
CARCINOID LUNG(1%)
DM+HT(3%)

Table 6 : PAST H/O TB AND TREATMENT H/O

Past H/O TB	ATT Taken	Treatment Defaulter
Pulmonary(9)	7	2
Extra Pulmonary(3)	3	-
Total-12	10	2

Among the 12 patients who had past H/o tuberculosis 10 patients completed treatment and 2 patients defaulted.

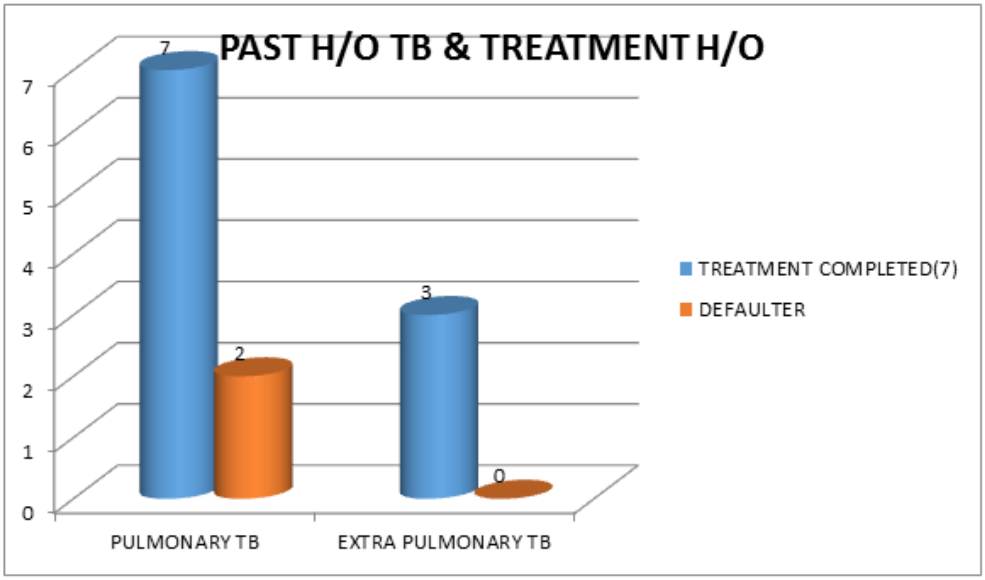


Table 7 : INVESTIGATIONS PROFILE FOR SYMPTOMATIC SMEAR NEGATIVE PULMONARY CASES.

Patients with	Peripheral Smear		HIV Status	
	Lymphocytosis (>70%)	Normal Count	Positive	Negative
Sputum (40)	21(42%)	17(38%)	1	39
BAL Fluid (9)	5	4	-	9

Out of 49 tuberculosis suspected patients 21(42%) patients had peripheral lymphocytosis and one(2%) patient was HIV positive.

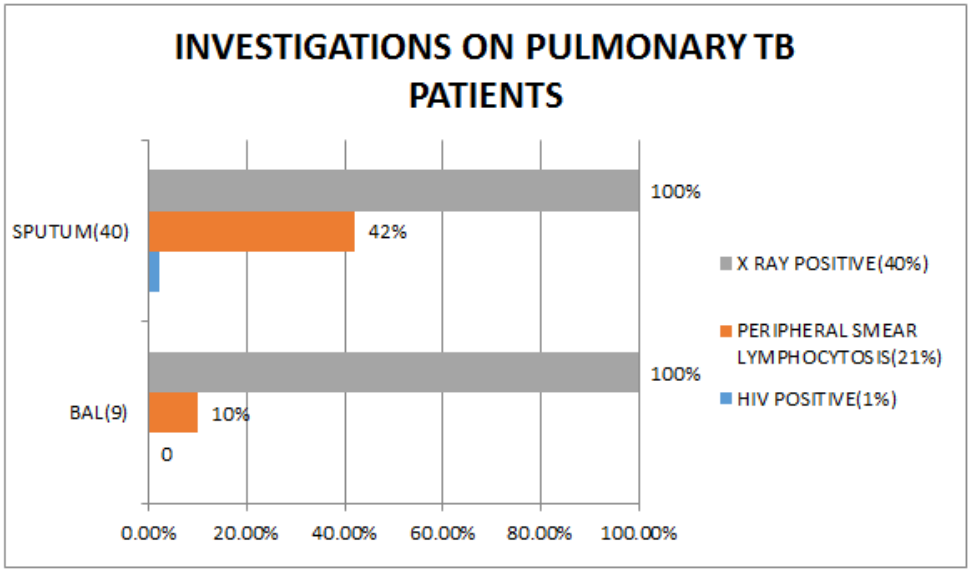


Table 8 : INVESTIGATIONPROFILE FOR SYMPTOMATIC EXTRA PULMONARY CASES

Patients with		Lymphocyte Count in Fluid (>70)		ADA (>50 U/L)		HIV Status		FNAC	
		wnl	Inc	wnl	inc	+ ve	-ve	+ve	-ve
Pleural Fluid (31)		13	18	17	16	1	30	-	-
AsciticFluid (5)		3	2	4	1	-	5	-	-
Pus (9)	Empyema(7)	-	-	-	-	1	8	-	-
	Psoas Abscesses(2)								
Sinus tract / wound discharges(3)		-	-	-	-	-	-	-	-
Lymph Node Aspirate (3)		-	-	-	-	-	3	3	-

wnl – Within Normal Limits, inc – Increased, +ve – Positive,

-ve – Negative.

18 patients had increased lymphocytosis and 16 had increased ADA count in pleural fluid. All 3 of the lymph node aspirates were positive for tuberculosis by Fine Needle Aspiration Cytology.

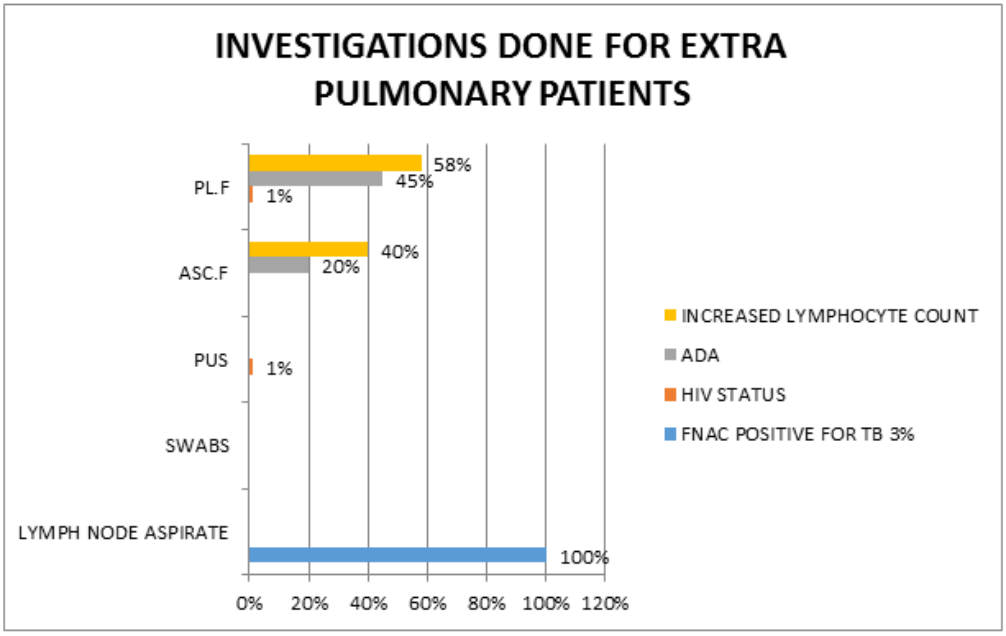
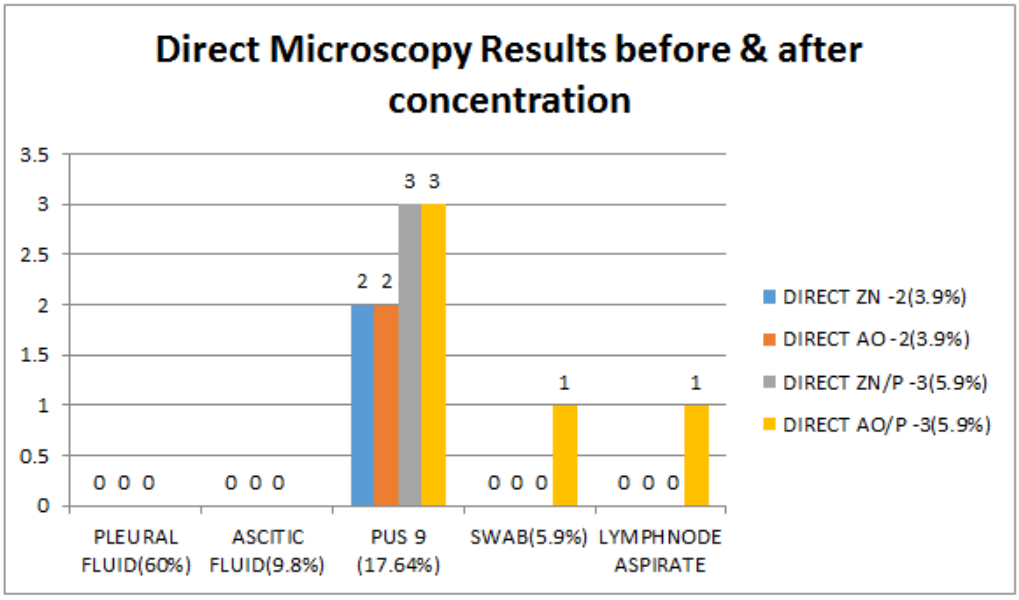

INVESTIGATIONS DONE FOR EXTRA PULMONARY PATIENTS
PL.F
ASC.F
PUS
SWABS
LYMPH NODE ASPIRATE
58%
45%
1%
40%
20%
1%
100%
0%
20%
40%
60%
80%
100%
120%
INCREASED LYMPHOCYTE COUNT
ADA
HIV STATUS
FNAC POSITIVE FOR TB 3%

Table 9 : DIRECT MICROSCOPY BY MODIFIED ZIEHLNEELSEN STAINING &AURAMINE O STAINING IN EXTRA PULMONARY SPECIMENS.

Extra pulmonary specimens n=51		Before concentration n= 51		After Concentration n=51	
		Ziehl Neelsen Stain	Auramine O Stain	Ziehl Neelsen Stain	Aura mine O Stain
Pleural Fluid(31)		-	-	-	-
Ascitic Fluid(5)		-	-	-	-
Pus(9)	Empyema(7)	2 (3.9%)	2 (3.9%)	3 (5.9%)	3 (5.9%)
	Psoas Abscesses(2)				
Wound Discharges(3)	Sinus tract Discharge(2)	-	-	-	1(1.8%)
	Ulcer(1)				
Lymph node Aspirate(3)		-	-	-	1 (1.8%)
Total		2	2	3	5(9.8%)



AuramineO staining after concentration detected 5(9.8%) samples.

Ziehl-Neelsen staining detected 2(3.9%) samples before concentration and detected 3(5.9%) samples after concentration.

There is no difference in the detection of modifiedZiehl-Neelsen&Auramine O staining methods before concentration.

Sensitivity of processed Auramine O staining after concentration for Extra pulmonary specimens is 30% & specificity is 97%.Overall PPV is 87.3% and for Extra pulmonary specimens it was 80% in this study.

Table 10 : CULTURE POSITIVITY BY MGIT IN SMEAR NEGATIVE PULMONARY & EXTRA PULMONARY SPECIMENS.

Category n=100	Specimen		MGIT +ve		MGIT -VE	CONTAMINATED CULTURES
			MTBC	MOTT		
Pulmonary (49)	Sputum (40)		3	3	30	4
	BAL Fluid (9)		-	2	7	-
PUL-Total	**49**		**3**	**5**	**37**	**4**
Extra pulmonary (51)	Pleural Fluid(31)		1	1	27	2
	Ascitic Fluid (5)		-	-	5	-
	Pus(9)	Empyema(7)	3	1	2	2
		Psoas Abscess(2				
	Sinus Tract/Wound ischarge (3)		1	-	2	1
	Lymph Node Aspirates(3)		2	-	1	-
EXT PUL-Total	**51**		**7**	**2**	**37**	**5**
TOTAL	**100**		**10**	**7**	**74**	**9**

MTBC- Mycobacterium tuberculosis complex.

MOTT-Mycobacterium Other Than Tuberculosis Complex,

Pul-Total- pulmonary Total, EXT- Total- Extra pulmonary Total

Recovery rate for extra pulmonary specimens was 9/51(18.5%). Among Extra pulmonary specimens, the positivity was higher for pus specimens out of 9/51(18%) specimens it detected 5/9(55.6%).

Out of 100%, 9% of cultures were contaminated with gram positive organisms & 74% of them were negative.

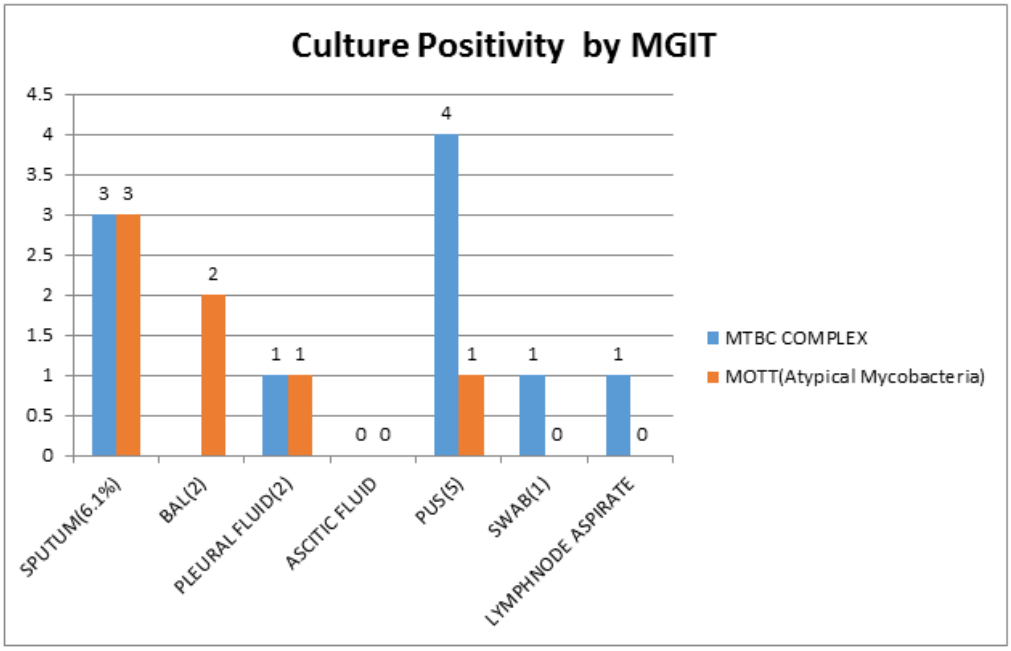


Table 11 : ANTIMYCOBACTERIAL SENSITIVITY TESTING RESULTS BY MGIT

		Streptomycin		Isoniazid		Rifampicin		Ethambutol	
		Sen	**Res**	**Sen**	**Res**	**Sen**	**Res**	**Sen**	**Res**
Sputum(6)	MTBC (3)	3	-	3	-	3	-	3	-
	MOTT (3)	-	3	-	3	-	3	-	3
BAL fluid(2)	MOTT (2)	-	2	-	2	-	2	-	2
Pleural Fluid(2)	MTBC (1)	1	-	1	-	1	-	1	-
	MOTT (1)	-	1	-	1	1	-	-	1
Pus(5)	MTBC (4)	4	-	4	-	4	-	4	-
	MOTT (1)	-	1	-	1	-	1	-	1
Sinus Tract Discharge (1)	MTBC (1)	1	-	1	-	1	-	1	-
Lymph node aspirate(1)	MTBC (1)	1	-	1	-	1	-	1	-

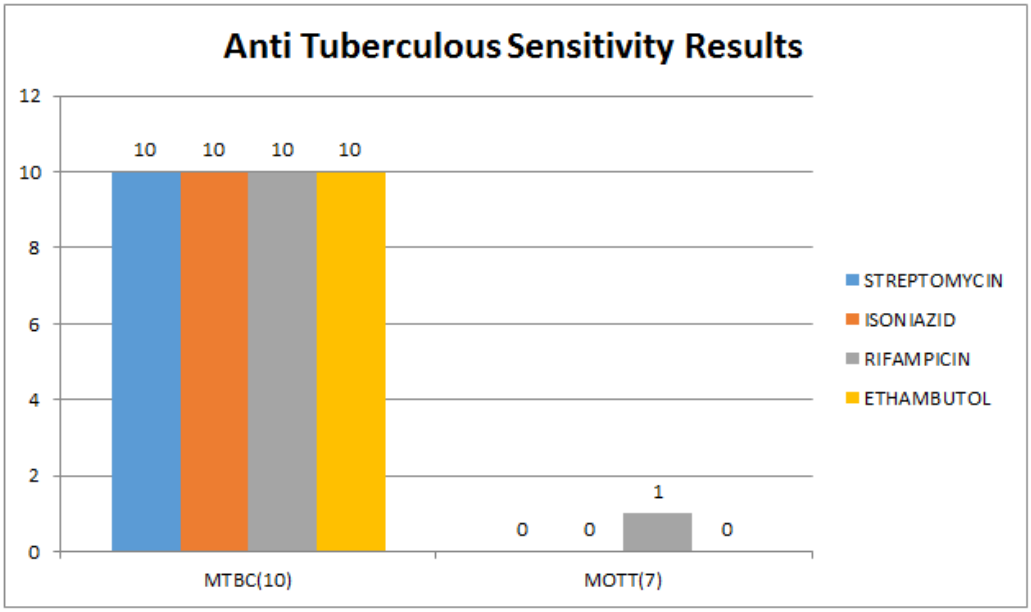
MTBC- MYCOBACTERIUM TUBERCULOSIS COMPLEX,

MOTT-MYCOBACTERIUM OTHER THAN TUBERCULOSIS.

Sen-Sensitive,Res-Resistant

All 10/17 of MTBC strains were sensitive to all four drugs Streptomycin, Isoniazide, Rifampicin, Ethambutol.

Among the 7/17 of MOTT all where resistant to three drugs streptomycin , Isoniazide, Ethambutol, except one strain which was sensitive to rifampicin.



ATYPICAL MYCOBACTERIA RESISTANT TO ALL(MGIT)

(MGIT) Table 12 : SPECIMEN WISE GENE X PERT RESULTS AND RIFAMPICIN SENSITIVITY.

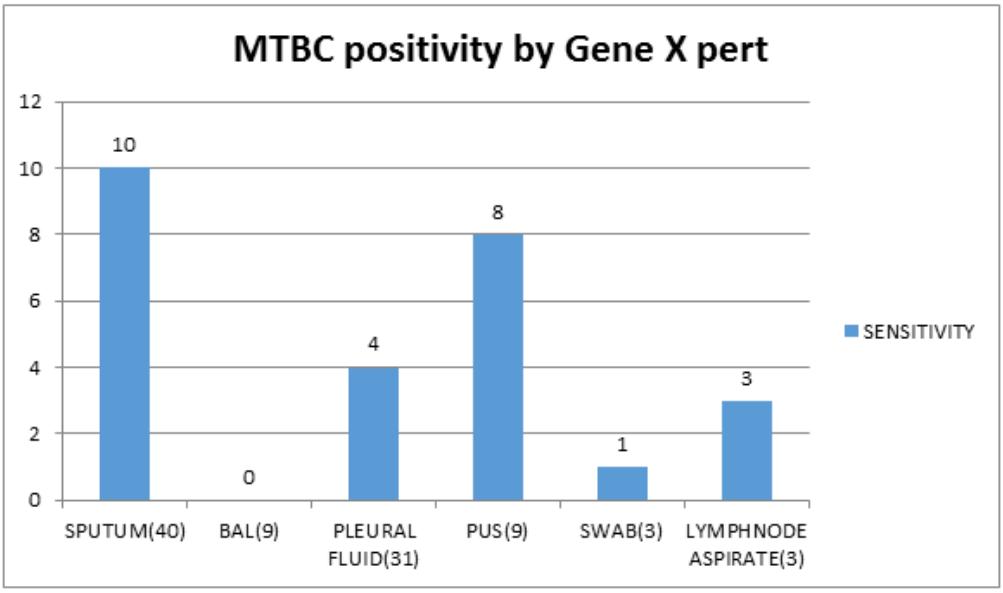
Category n =100	Specimen		Positive	Negative	Invalid/ Error	Rifampicin	
						S	R
Pulmonary (49)	Sputum(40)		10	28	2	9	1
	BAL(9)		-	9	-	-	-
Pul-Total	**49**		**10**	**37**	**2**	**9**	**1**
Extra pulmonary	Pleural Fluid (31)		4	27	-	4	-
	Ascitic Fluid (5)		-	5	-	-	-
	Pus(9)	Empyema(7)	8	1	-	8	-
		Psoas abscess(2)					
	Wound Discharges (3)		1	2	-	1	-
	Lymph Node Aspirate(3)		3	-	-	3	-
Ext Pul - Total	**51**		**16**	**35**	**-**	**16**	**-**
Total	**100**		**26**	**72**	**2**	**25**	**1**

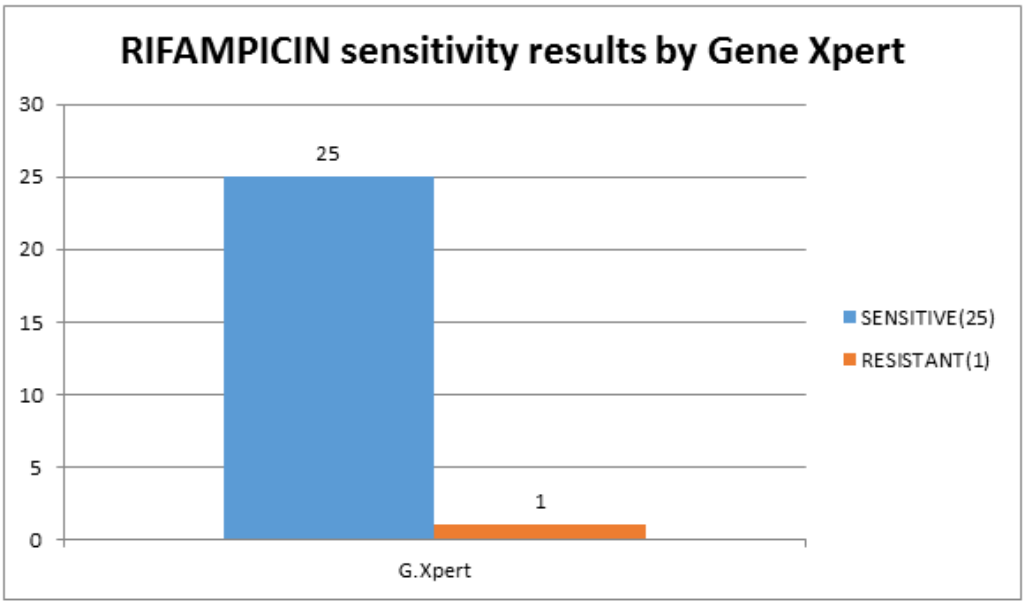
S- sensitive, R-Resistant, Pul- pulmonary,Ext- Extra pulmonary.

In total GENE X pert /RIF detected 26%, in Smear Negative Pulmonary samples it detected 10/49(20%). It detected 16/51(31%) in extra pulmonary specimens.

Among the 9 pus samples it detected 8/9(89%).In lymph node aspirates out of 3 specimens it detected all the 3/3(100%).

Out of 26 % of positivity, one strain wasRifampicin resistance, rest all were sensitive.


MTBC positivity by Gene X pert
12
10
8
6
4
2
0
10
0
4
8
1
3
SPUTUM(40)
BAL(9)
PLEURAL FLUID(31)
PUS(9)
SWAB(3)
LYMPHNODE ASPIRATE(3)
SENSITIVITY


RIFAMPICIN sensitivity results by Gene Xpert
30
25
20
15
10
5
0
25
1
G.Xpert
SENSITIVE(25)
RESISTANT(1)

13. TIME OF DETECTION OF POSITIVE SAMPLES BY MGIT.

Category	MGIT In Days						
	Time Of Detection(Days)	<5	6 - 10	11 - 15	16 - 20	21- 25	26 - 42
Pulmonary	Sputum (6)	1	3	1		1	
	Bronchio Alveolar Fluid(2)	1	1				
Extra Pulmonary	Pleural Fluid(2)	1		1			
	Ascitic Fluid						
	Pus(5)	1	2	1		1	
	Swab(1)		1				
	Lymph Node Aspirate (1)		1				
	Total	4	8	3	-	2	-

The mean time of detection of MGIT was 9.6 days.

The mean time of detection for MOTT is just 5.8 days.

The mean time of detection for pulmonary specimens is 9.3 days.

The mean time of detection for Extra pulmonary specimens is 9.8 days.

The mean time of detection of MOTT is very less 5.8 days compared to *Mycobacterium tuberculosis* complex. Gene X pert detected all

samples in less than 2 hours.

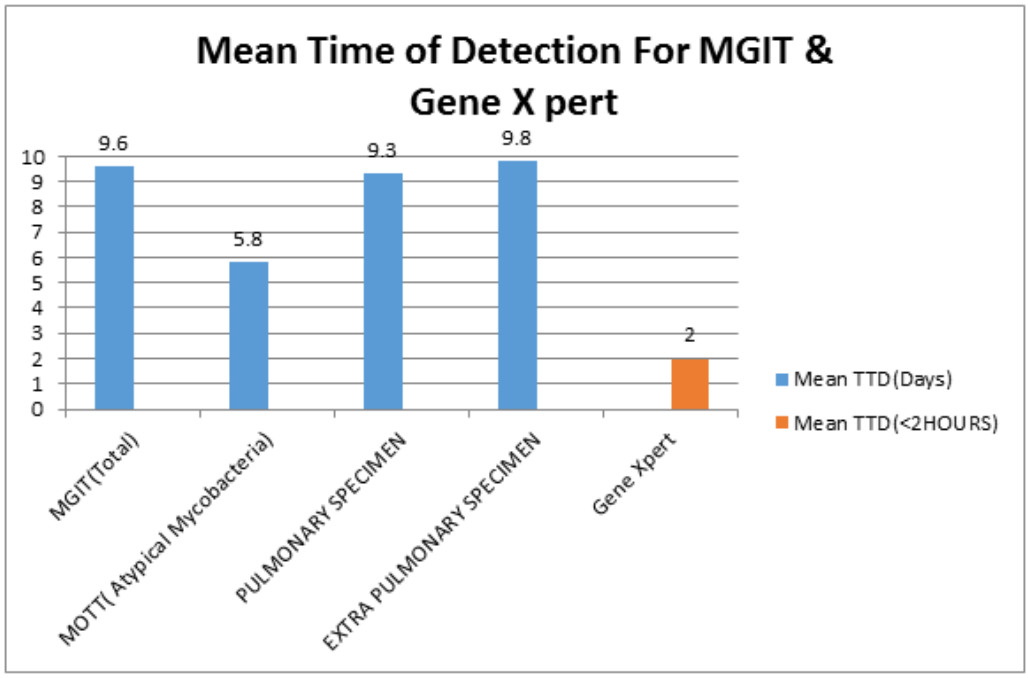
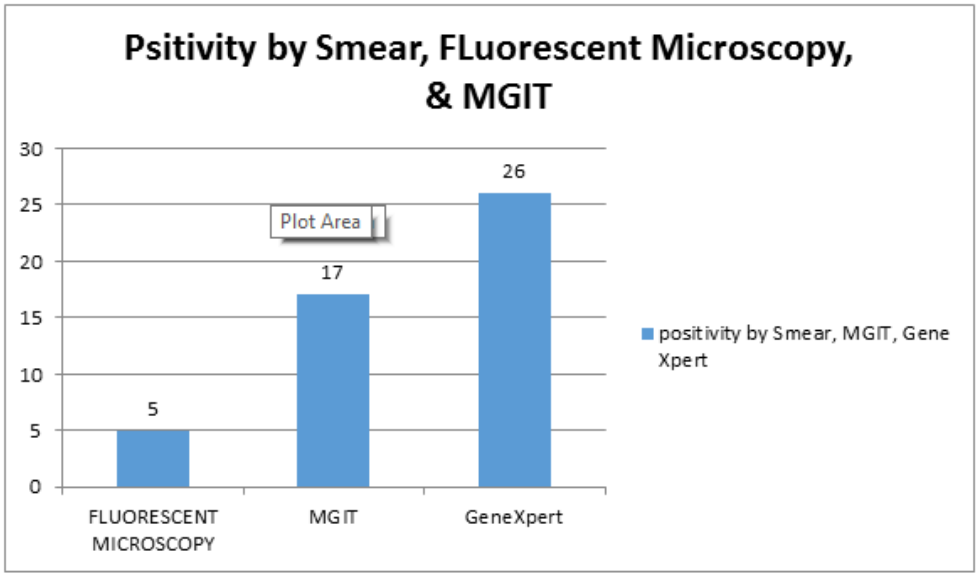


Table 14 : POSITIVITY BY FLUORESCENT MICROSCOPY, MGIT and Gene Xpert

Detection method	Number positive	%
Fluorescent Microscopy	5	5%
MGIT	17	17%
Gene X pert	26	26%

Gene X pert detected maximum by 26%, secondly MGIT 17% and last Fluorescent Staining 5%.



Comparison of MTB COMPLEX positivity by MGIT & Gene Xpert in relation to smears.

Table 15 : COMPARISON OF POSITIVITY BY MGIT CULTURE & GENE X PERT, IN RELATION TO FLUORESCENT MICROSCOPY.

Category	Specimen		FLUORESCENT /MICROSCOPY	MGIT POSITIVE		Gene Xpert Positive
				MTBC	MOTT	
Smear Negative Pulmonary	Sputum (40)		-	3	3	10
	BAL (9)		-	-	2	-
PulTotal	**49**		**-**	**3**	**5**	**10**
Extra Pulmonary	Pleural Fluid		Negative (31)	1	1	4
	Ascitic Fluid		Negative (5)	-	-	-
	Pus	Psoas abscesses	Positive (3)	3	-	3
		Empyema	Negative (6)	1	1	5
	Wound Discharges		Positive (1)	1	-	1
			Negative (2)	-	-	-
	Lymph Node Aspirate		Positive(1)	1	-	1
			Negative(2)	-	-	2
Ext Pul Total			**Positive-5**	**7**	**2**	**16**
Total			**5**	**10**	**7**	**26**

Pul-Pulmonary, Ext Pul- Extra pulmonary.

Sensitivity ,specificity, PPV, NPV of Fluorescent Microscopy is 29.4%,100%,87.3%,100%.

Overall including MTBC& MOTT the sensitivity & specificity of Gene X pert is 60% and 78%.

Table 16 :RECOVERY RATE OF MYCOBACTERIUM FROM CLINICAL SAMPLES BY MGIT & GENE X PERT IN RELATION TO SMEARS.

S.No	MGIT			Gene X Pert(MTB Complex)
	MTB Complex	MOTT	MGIT TOTAL	
Smear Neg Pulmonary (49)	3(6.1%)	5(10.2%)	8(16.3%)	10
Smear Pos Extra Pulmonary (5)	5(100%)	-	5(100%)	5
Smear Neg Extra Pulmonary (46)	2(4.3%)	2(4.2%)	4(8.6%)	11
TOTAL	**10**	**7**	**17**	**26**

In 49 smear negative pulmonary specimens MGIT detected 8/49(16.3%).Out of which 5 (10.2%) were MTBC & 3(6.1%) were MOTT.Gene X pert detected 10/49 (20%).

Out of 5 smear positive Extra pulmonary specimens MGIT& Gene X pert detected 5/5(100%) all belongs to MTBC .There was 100 % agreement

In smear negative Extrapulmonary specimens 46/51MGIT detected 4/46(8.7%) among them 2(4.3%) MTBC ,&2(4.3%) were MOTT , Gene X pert detected 11/46(24%) and it also detected extra 7/46

(15.2%).

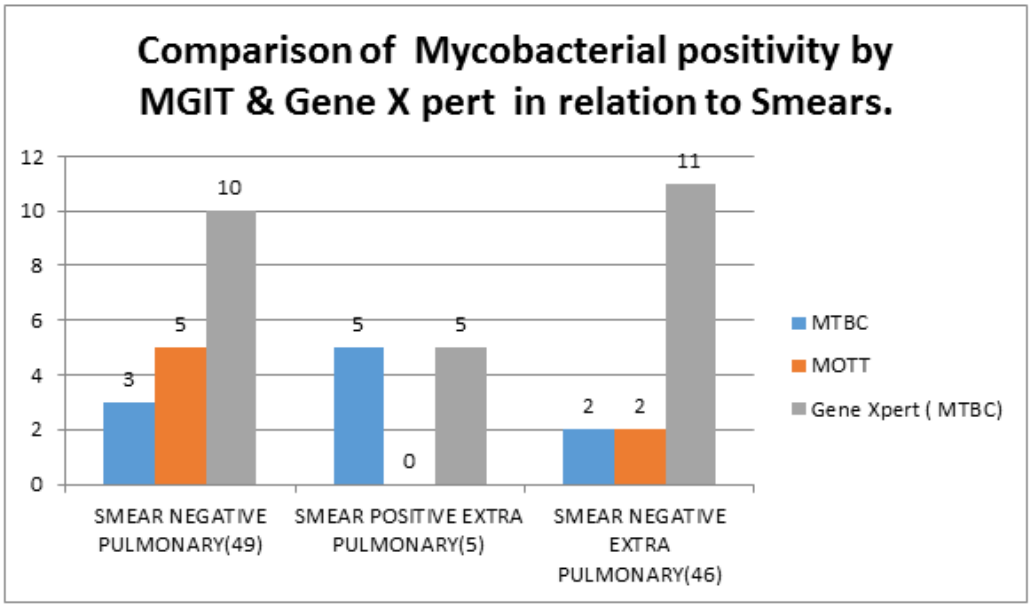
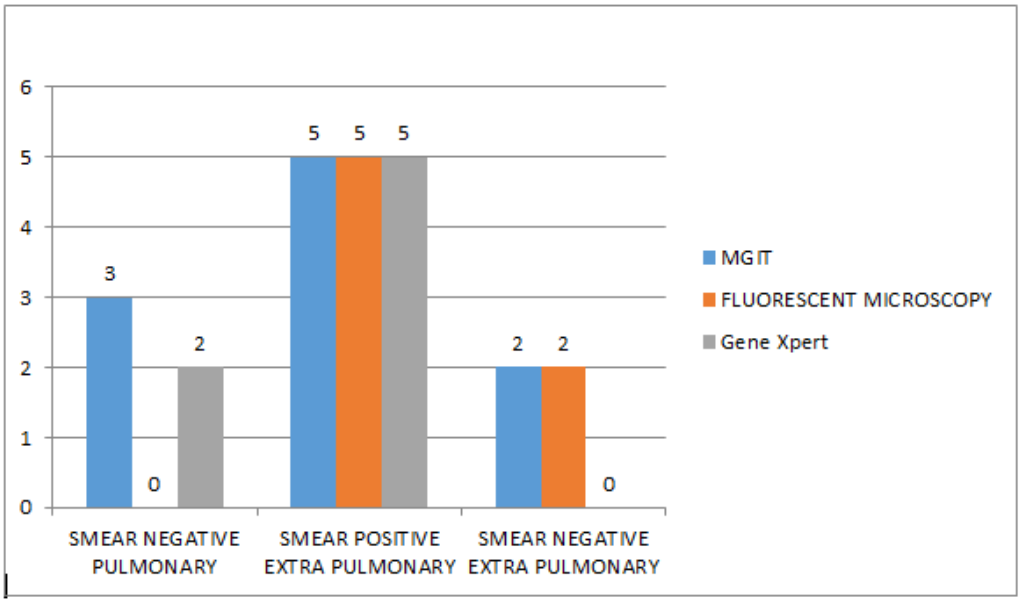
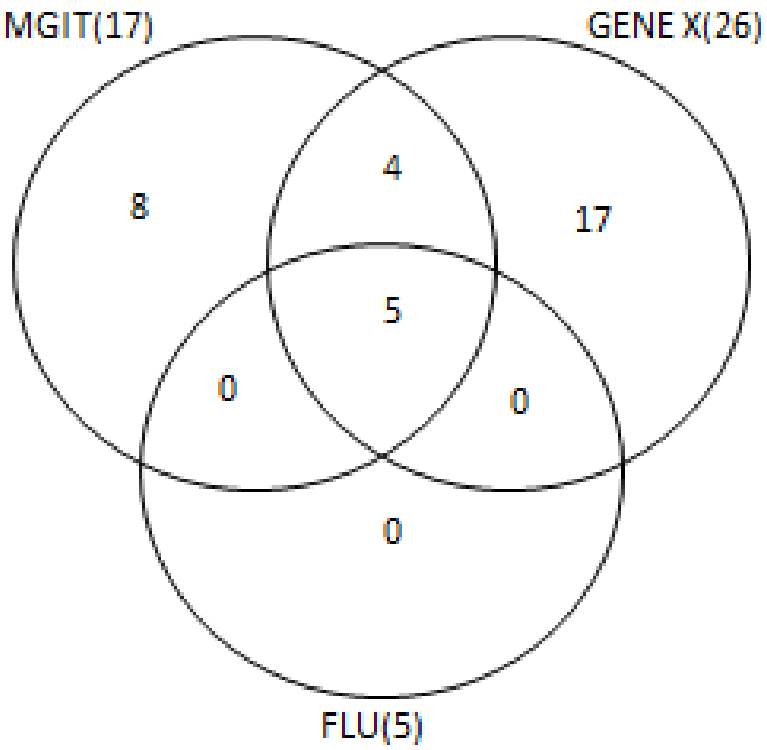

Comparison of Mycobacterial positivity by MGIT & Gene X pert in relation to Smears.
12
10
8
6
4
2
0
3
5
10
5
0
5
2
2
11
SMEAR NEGATIVE PULMONARY(49)
SMEAR POSITIVE EXTRA PULMONARY(5)
SMEAR NEGATIVE EXTRA PULMONARY(46)
MTBC
MOTT
Gene Xpert (MTBC)

Table 17 :POSITIVITY OF GENE X PERT & FLUORESCENT MICROSCOPY IN RELATION TO MTB COMPLEX MGIT CULTURE POSITIVITY.

Specimens n=10	MGIT, MTb Complex Culture Positive	Fluorescent Microscopy Positive	Gene X Pert Positive
Smear negative pulmonary(3)	3	-	2
Smear positive Extra pulmonary TB(5)	5	5	5
Smear negative Extra pulmonary TB(2)	2	-	2
TOTAL(10)	10	5	9



Out of 10 of MGIT positive MTB complex cultures, Gene X pert detected 9 (90%) ,so there is 90% agreement.



MGIT POSITIVE ,GENE X PERT NEGATIVE-1

GENE X PERT POSITIVE, MGIT NEGATIVE-17

MGIT AND GENE XpertPOSITIVE- 9

Table 18 : COMPARISON OF MTB COMPLEX SENSITIVITY RESULTS BY MGIT & GENE X PERT.

	MGIT		GENE X PERT	
	SEN	**RES**	**SEN**	**RES**
STREPTOMYCIN	9	-	NA	**-**
ISONIAZIDE	9	-	NA	**-**
RIFAMPICIN	9	-	9	**-**
ETHAMBUTOL	9	-	NA	**-**

100 % AGREEMENT

There is no discrepancy in antibiotic susceptibility results by both MGIT & Gene X pert out of 9 samples detected by both methods.

DISSCUSSION

According to WHO report 2012 there are 8.6 million new cases and 1.3 million TB deaths per year[1].In India out of one billion total population , around 2 million develop active TB and 50% of them die each year.

The present study was conducted to test & compare the efficiency and reliability of LED Fluorescent Microscopy, liquid culture medium(MGIT), & GENE X pert for the detection of Mycobacteriumtuberculosisin smear negative pulmonary specimens and extra pulmonary specimens, and to do anti tuberculous sensitivity pattern by Mycobacterial Growth Indicator Tube.

Totally 100 patients suspected of having tuberculosis infection were included in this study ,among them sputum &Bronchio- alveolar lavage fluid were collected from 49 patients, pleural fluid(31) ,ascitic fluid(5), pus/empyma(7), Psoas abscesses(2), Breast abscess with sinus tract discharges/wound discharges(3), lymph node aspirates(3) were collected from 51 patients and processed.

Table-1 : Age Distribution:

.Among the tuberculosis suspected patients the commonest age group was 41 to 60 years, and the mean age was 56.3years in this study which correlates with the study conducted by Salome[64]Phelamei et al in which the age group affected was 15 to 54 yrs.

Table 2 : Gender distribution in pulmonary and extra pulmonary specimens

Among the tuberculosis suspected patients 74% were men and 26% were women, this is highly significant, and they were equally distributed in both pulmonary and extra pulmonary category.This correlates with study of Mohamed[35] et al where 78% of men and 22% of women affected[35].According to Olivier[65] et al because of unique sex specific genetic architecture in men, they have a complex of genes HLA class I ,NRAMP 1 and TLR which makes them susceptible to Tuberculosis.But in females oestradiol enhances macrophage activation, which in turn increases TNF α ,which provides protection.

Table 3 : SOCIO ECONOMIC STATUS ACCORDING TO KUPPUSAMY CRITERIA.

In this study most of the patients belongs to lower middle class(57).

This correlates withDeeraj kumar[63] et al, in which patients with lower socio- economic status has a higher risk of tuberculosis.Because in people with lower socio- economic status, the literacy rate is poor which leads to poor knowledge of TB, risks of infection and delayed approach to health care facility. Poverty leads to poor nutrition and it suppress the immune system, and makes people vulnerable to tuberculosis[62].

Table 4 : Presenting complaints.

The commonest complaint was constitutional symptoms 62%. It is due to elevation of Lymphokines and cytokines such as IL12, TNF α,and TGF β[65] which correlates with the study of S.K Sharma [7] et al , where constitutional symptoms was the major complaint in Extra pulmonary tuberculosis patients. The second commonest complaint in this study was breathlessness in extra pulmonary & Hemoptysis in pulmonary Tuberculosis suspected patients.

. **Table 5 : ASSOCIATED DISEASES.**

Most common associated diseases among the tuberculosis suspected patients was Diabetes Mellitus(7%) followed by carcinomas(4%).This correlates withsalome[64] et al, where Diabetes Mellitus was the most common associated disease, which is explained due to immunosuppression and its vulnerability to infection.

Table 6 : PAST H/O TB AND TREATMENT H/O

Among the 12 patients who had past H/o tuberculosis 10 patients completed the treatment and 2 patients defaulted, because of not knowing the complications of tuberculosis.

.Table 7 : INVESTIGATIONS PROFILE FOR SYMPTOMATIC SMEAR NEGATIVE PULMONARY CASES.

Out of 49 tuberculosis suspected patients 21(42%) had peripheral lymphocytosis, which is similar to [50.] and 1(2%) was HIV positive

Table 8 : INVESTIGATIONPROFILE FOR SYMPTOMATIC EXTRA PULMONARY CASES

Among the 51 Extra pulmonary tuberculosis suspected patients,18 had increased lymphocytosis,16 had increased ADA(.50U/L) count in pleural fluid. This correlates with Sven O Friedrich et al and Verma et al in which there was a strong association of increased Adenosine de aminase levels.All 3 of the lymph node aspirates were positive for tuberculosis by Fine Needle Aspiration Cytology.

Table 9 : Direct Microscopy by Modified ZiehlNeelsen staining &Auramine O staining in Extra pulmonary specimens.

Among the 51 Extra pulmonary specimens LED Fluorescent Microscopy by AuramineO stainingafter concentration

detectedmaximum 5(9.8%) samples**,** this correlates with Gerardo[25] et al in which positivity of LED microscopy in EPTB was 9.1%.In this study all the 5(9.8%) positive smears were pus/empyma, Psoas abscesses and lymph node aspirates which constitutes 5/12(40%) this is more or less similar toSiddiqui[25]et al in which lymphnode aspirates showed higher percentage of positivity(33%)

In the present study Ziehl-Neelsen staining detected 2(3.9%) samples before concentration and 3(5.9%) samples after concentration. This correlates with Siddiqui[25] et al in which 5% were positive by ZN staining in EPTB.

In this study there was no difference in the detection rate of Ziehl-Neelsen& AO staining methods before concentration. The cumulative& valid percentage of detection is 3.9 for both, there is no statistical significance(0.735) with 95% Confidence Interval. According to Rohi[11] et al, ZN staining detected only 2% which exactly correlates with the present study.

The sensitivity, specificity and positive predictive value of Fluorescent Microscopy forExtra pulmonary specimensinthe present study was 29.4%,100%,80% respectively.

Table 10 : Culture Positivity By MGIT in Smear Negative Pulmonary & Extra Pulmonary Specimens.

In this study MGIT(Mycobacterial Growth Indicator Tube) detected 17 among which 10/17(58.8%) where Mycobacterium tuberculosis complex and 7/17(41%) where MOTT (Atypical mycobacteria).

Among the 49 smear negative pulmonary specimens, MGIT detected 8/49(16.3%) in which M.tuberculosiscomplex was 3 and MOTT was 5,MGIT detected more atypical mycobacterias compared to Mycobacterium tuberculosis complex.This correlates with Mohamed[35] et al in which the positivity in Smear negative specimens where 16%, and by Gaby [36] et al it was 14%.

In this studyoverall atypical mycobacterial detection was 7/17(41%) , but in smear negative pulmonary specimens, it was5/8(62%) which is less than Gaby[14]et al where MGIT recovered 39% of Atypical Mycobacterias.

The recovery rate of MGIT for extra pulmonary specimens was 18% in the present study, this correlates with the Arzu[22] et al of 17.6%.this is higher than Hilleman[8] et al in which recovery rate was 7.8%.

Among Extra pulmonary specimens, the positivity was higher for pus/empyema, Psoas abscesses and lymph node aspirates,out of 9(18%) specimens it detected 5(55.6%).

All the atypical mycobacterias were subcultered into Lowestein Jensen medium, among which 1 specimen produced pink colored pigment in the presence of light and so this was confimed as photochromogen, rest 6/7 samples showed growth in less than 7 days and this was confimed as rapid growers.

9% of cultures were contaminated with gram positive organisms, in this study this is similar with Gaby[14] 6-8 % &Mohamed et al 6.1%, Jacob[37] et al 6.8 % .To rule out bacterial and fungal contamination , positive MGIT tubes where subcultured into Blood Agar Plate, Macconkey Agar Plate and SDA(Sabouraud Dextrose Agar).

Table 11 : ANTIMYCOBACTERIAL SENSITIVITY TESTING RESULTS BY MGIT

Out of 17 MGIT positives, all 10/10(100%) of MTBC strains were sensitive to all four drugs Streptomycin(1.0µg/ml), Isoniazide (0.1µg/ml), Rifampicin(1.0µg/ml), Ethambutol(5.0µg/ml) which correlates with Palicova[18]et al the accuracy of MGIT was acceptable(>95%)

ATYPICAL MYCOBACTERIA WITH PIGMENT

SENSITIVE TO RIFAMPICIN , RESISTANT TO OTHERS

Among the 7 MOTT(Atypical mycobacteria) all were 100 % resistant to three drugs streptomycin(1.0µg/ml) , Isoniazide(0.1µg/ml), Ethambutol(5.0µg/ml)., and only one sample was sensitive to Rifampicin(1.0µg/ml). This correlates with the study done by Gomathi[16] et al , in which accuracy was >92%.

Table 12 : SPECIMEN WISE GENE X PERT RESULTS AND RIFAMPICIN SENSITIVITY:

GENE X pert /RIF detected 26% the sensitivity, specificity and positive predictive valve(PPV) of the MTB/RIF test was 60%,77% and 97% this correlates with a study conducted by Rishi[21] et al in which sensitivity was 70%, and the PPV was 100%.

In this study among the 49 pulmonary specimens , the detection rate was (10/26) 20%, and the sensitivity and specificity of the MTB/RIF test were50%, 80% respectively , which is less than 82.3% and 100% respectively according to Rishi et al, which may be because of the inclusion of smear positive pulmonary specimens.

Gene X pert detected 16/51(31.3%) of extra pulmonary specimens,which correlates with a study conducted by Arzu[21] et al where 32.1%. The sensitivity & specificity of extra pulmonary specimens was 75% & 76% this reports were higher than Rishi et al in which, overall sensitivity of extra pulmonary specimens was 52.1%.

Among the 9 pus samples it detected 8/9(89%).In lymph node aspirates out of 3 of specimens it detected all the 3/3(100%), this correlates with panayotis[24] et al in which positivity in tissue samples was 70-80%.

Out of 3/100 (3%) of wound discharges , 1 sample was positive, rest of the 2 samples which came negative by Gene X pert/ RIF assay was further followed up , and the FNAC report suggested of having carcinomatous changes.

In this study the positivity was very less in pleural fluid among 31/51(61%) detected only 4 /31(13%) , this is higher than the Hillemann et al found 3% positive, and Causse[9] et al 4/34(11.8%) positive , In a study by Sven[23] et al sensitivity is only 25 % but specificity is 100% for pleural fluid.In pleural&Ascitic fluid samples, the positivity is less, this could be due to the presence of aninhibitory substance which inhibited the amplification of the M. tuberculosis genome[21].

The results came invalid /error in 2% of pulmonary samples , 98% of specimens gave interpretable results, one was insufficient sample and another came as error, this correlates with the studies like Sven O [23]et al and Panayatis[24] et al where uninterpretable results was 1.8% and 2.4% respectively.Gene X pert detected all samples in less

than 2 hours. This test detects mycobacteria very rapidly < 2hrs compared to Gold standard culture methods like LJ in which mean time of detection is (31±9.4 days)[35] and for MGIT (9 ± 5 days),

Out of 26 % of positivity, Rifampicin resistance was detected in only one 1% of sputum sample, rest all were sensitive.

13. TIME OF DETECTION OF POSITIVE SAMPLES BY MGIT.

. The mean time of detection of MGIT was 9.6 days, in this study.However in different studies, time to detect Mycobacteria by MGIT falls in the range of (11.6-14.4 days) according to Gaby [14] et al.

The mean time of detection for pulmonary specimens is 9.3 days. For smear- negative specimens, it was 14.9 days according to Haung[35] et al.

The mean time of detection for MOTT(*Atypical Mycobacteria*) by MGIT is just 5.8 days. This is statistical significance ($p < 0.05$), which is similar to Gaby [36] et al in which it is less than 7 days.

The mean time of detection for Extra pulmonary specimens is 9.8 days. There is no statistical significance ($p>0.05$) between mean time of detection in smear negative pulmonary and extra pulmonary specimens .

This correlates with the study of Mohamed [35] et al and Pfyffer et al , where the mean time of detection was 9.9 days in pulmonary specimens.which correlates with the present study. Growth of M.tuberculosis complex in smear positive and smear negative specimens occurred in the MGIT after 5 days .

Table 14 : POSITIVITY BY FLUORESCENT MICROSCOPY, MGIT and Gene Xpert

In total out of 100 specimens , Fluorescent Microscopy detected 5, MGIT detected 17 and Gene Xpert detected 26%. This correlates with the study of Panayotis[23] et al in which the positivity was 33.9%. compared to Microscopy & MGIT culture Gene X pert detected the maximum this is because Xpert assay is seminested real time PCR , it can amplify even a single DNA copy, in contrast to f MGIT Culture in which ,atleast 100 viable bacilli is essential.

Table 15 &16 :RECOVERY RATE OF MYCOBACTERIUM FROM CLINICAL SAMPLES BY MGIT & GENE X PERT IN RELATION TO SMEARS.

In smear negative pulmonary specimens out of 49/100(49 %) ,MGIT detected 8/49(16.3%) in which 3(6.1%) were MTBC and 5 (10.2%) were MOTT atypical *Mycobacteria.*Gene X pert detected 10 /49 (20%) of smear negative specimens , which is higher than MGIT.

In smear positive Extra pulmonary specimens out of 5% both MGIT & Gene X pert detected 5/5(100%) and more over all of them belongs to Mycobacterium tuberculosis complex. This correlates with Badak[35] et al MGIT positivity in smear positive specimens is 98%, & Pfyffer[35] et al it was 88.5%.

In smear negative Extrapulmonary specimens out of 46/51 ,MGIT detected 4(8.7%) among them 2(4.3%) belongs to Mycobacterium tuberculosis complex ,&2(4.3%) belongs to MOTT atypical Mycobacteria. Gene X pert detected 11/46(24%) which in turn detectedextra 7/46 (15.2%)MTBC strains. 2 MTBC strains of MGIT were also detected by Gene X pert.MOTT was not detected.

Overall in smear negative extra pulmonary specimens the detection rate is 11/46(%), this correlates with Doris Hilleman[11]et al and Arzu[22] et al in but in smear-positive extrapulmonary specimens positivity was 100% (4/4) and 37.7% (21/44) for smear-negative extrapulmonary specimens.

Table 16 :RECOVERY RATE OF MYCOBACTERIUM FROM CLINICAL SAMPLES BY MGIT & GENE X PERT IN RELATION TO SMEARS.

Among the 10 Mycobacterium tuberculosis complex strains, Fluorescent Microscopy detected only 5/10(50%) , and Gene X pert detected 9/10 (90%). This correlates with Zeka[21] et al in which

positivity of Gene Xpert in smear negative and smear positive specimens in relation to MTBC culture strains was 57 to 70%, and 98-100 % respectively. The main disadvantage of Gene X pert is that, it can detect only MTBC, it is not designed to detect MOTT, because this does not have the primer for MOTT .

VEN DIAGRAM : 18

In total the positivity of Gene X pert, excluding MOTT is 26/93(28%) ,in tuberculosis suspects , which is lower than the detection rate of Sven [23] et al , this may be due to inclusion of higher number ofpleural &ascitic fluid, which constitutes 36/51(70 %) in extra pulmonary specimens, as reported in different studies ,Sensitivity of Gene X pert is very low ranges from (5-25%) for pleural &ascitic fluids ,positivity is on the lower side.

A) Both MGIT + Gene X pert = 9/10.

MGIT & Gene X pert commonly detected 9/100 specimens , X pert missed one sample ,.

B) MGIT alone positive , Gene X pert negative = 1.

The reason could be the mutated strain.

C) MGIT negative ,Gene X pert alone positive = 17.

Gene X pert is an Nucleic Acid Amplification technique which is more superior than culture , because a single DNA copy itself can get amplified, But to get the culture to be positive there should be atleast 100 CFU/ ml of specimen.

D)Gene X pert negative, MGIT positive = 8

As discussed , 7 was Atypical Mycobacteria & 1 was MTB complex, that could be a mutated strain .

Table 18. There is no discrepancy in the Rifampicin sensitivity results for the 10 Mycobacterium tuberculosis complex strains.

According to this study the positivity is higher in extra pulmonary specimens compared to pulmonary specimens , this may be because of inclusion of smear negative specimens. Although Fluorescent microscopy detected only 5/100(5%) of the specimens , there is 100 % agreement with the culture, all those five samples were positive by both MGIT culture & Gene X pert.

Among the 10MGIT Mycobacterium tuberculosis complex positive samples, LED Fluorescent Microscopy after concentration detected 5 /10(50%). All the smear positive specimens were detected by both MGIT culture & Gene X pert. Although the sensitivity was low the specificity is 100 % . Since the positivity is only 9.8% in extra

pulmonary specimens , it will not be very much helpful in detecting Extra pulmonary Tuberculosis and also it did not detect even a single smear negative pulmonary specimens even after concentrating the specimen.

Gene X pert is a Catridge based semi nested multiplex PCR technique which detects Mycobacterium tuberculosis complexand Rifampicin Resistancerapidly in less than 2 hours from the clinical specimens directly with a higher positives. Furthermore this assay does not need any expertise training or sophisticated laboratory, and it is just a desktop machine which can be installed anywhere even in peripheral labs.The disadvantages are expensiveness it is designed only for Mycobacterium tuberculosis complex detection, but to identify atypical mycobacterias MGIT culture may be used.

The Micro MGIT system was found to be more efficient in detecting mycobacteria in smear-negative& Extra pulmonary specimens compared to Fluorescent Microscopy

Mycobacterium tuberculosis complex(MTBC) detection was 12% by Micro MGIT by Ruhi bund[11] et al, and 8% by Hillemann[11] et al, both of which are less than this study..

SUMMARY

This comparative study was conducted to asses and compare the effectiveness of LED Fluorescent Microscopy, MGIT(mycobacterial Growth Indicator Tube) and Gene Xpert MTB/RIF assay, in smear negative pulmonary specimens and Extra pulmonary specimens from patients clinically and radiologically suspected of having *Mycobacterium tuberculosis*.

In total of 49 smear negative pulmonary and 51 extra pulmonary patients, specimens such as sputum, Bronchio alveolar Lavage fluid, Pus, wound disharge, Lymph node aspirates, pleural and ascitic fluids were collected from patients. All the specimens were processed according to Standard LaboratoryProcedures , to detect *Mycobacterium tuberculosis* .

Out of 100 specimens , LED Fluorescent Microscopy detected 5, and there was 100 % agreement with MGIT and Gene Xpert MTB/RIF.

MGIT culture detected 17/100(17%) in which 10/17(58%) were Mycobacterium tuberculosis complex and 7/17(41%) were Atypical Mycobacterias.

Gene Xpert MTB/RIF assay detected 26/100(26%) very rapidly in < 2 hrs.

In smear negative pulmonary specimens MGIT detected 8(16.3) with mean time of detection of 9.3 days, where Gene Xpert detected 10

Among the Extra pulmonary tuberculosis MGIT detected 9(18.5%) and Gene X pert detected 16(31%).

In particular the positivity was higher among Pus, lymph node aspirates and wound discharges. So Gene Xpert may be exclusively considered for tissue specimens . Even though the detection rate of X pert assay was less among pleural &ascitic fluids, it detected more positives compared to MGIT & LED Fluorescent microscopy.

Among the 10 MTBC culture positive strains Gene Xpert detected 9/10(90%).There was 90% agreement.

In total GeneXpert assay detected 4 times more positivity compared toLED Fluorescent Microscopy and 3 times more than the culture, excluding atypical mycobacteria. There was no discrepancy in the drug susceptibility testing by MGIT and Gene X pert in RIFAMPICIN resistance detection.

The disadvantages of Gene Xpert is that it is designed to detect only Mycobacterium tuberculosis complex and not Atypical Mycobacteria . The instrument is also too expensive.

According to this study, Atypical Mycobacteria constitutes 7/17(41%) among the positive cultures , so to identify atypical mycobacteria MGIT culture may be considered. LED Fluorescent microscopy & MGIT (Gold standard culture) can be implementedin places where Gene X pert is not affordable.

CONCLUSION

100 cases of smear negativepulmonary tuberculosis and smear positive and smear negative extra pulmonary tuberculosis suspected patients were included in the study. Specimens were collected and processed for LED Fluorescent Microscopy, MGIT and GeneXpert MTB/RIF assay.

Overall ,ModifiedZiehl- Neelsen detected 3,Fluorescent Microscopy detected 5, MGIT detected 17 and Gene Xpert detected 26.In smear negative pulmonary specimens MGIT detected 8 strains whereas Gene Xpert 10. Among the Extra pulmonary specimens ZN detected 3,Fluorescent Microscopy 5, MGIT detected 9, and Gene Xpert 16.

Compared to modified ZN staining ,Fluorescent Microscopy is superior which detected 5 .There was 100 % agreement between Fluorescent microscopy, in relation to MGIT culture and Gene Xpert. There was 90% agreement between MGIT & Gene X pert with respect to Mycobacterium tuberculosis complex identification.

Among the MTBC strains detected by MGIT, all were sensitive to Streptomycin, Isoniazid ,Rifampicin, &Ethambutol, which is also sensitive by Gene Xpert. There was no discrepancy in the sensitivity

results. Gene Xpert detected 1 MTBC Rifampicin Resistant strain which was not picked up by MGIT.Among the 7 Atypical mycobacterias all were resistant to Streptomycin, Isoniazide, and Ethambutol ,except one strain which was sensitive to Rifampicin by MGIT.

Although the cost of the equipment , cost per sample & maintenance charges are higher for Gene X pert it detects MTBC DNA & Rifampicin Resistance very rapidly in < 2 hrs directly from the clinical specimens compared to all other methods.

The results of this study indicated that the implementation of Gene Xpert MTB/RIF assay could dramatically improve the rapid diagnosis of tuberculosis particularly in smear negative pulmonary & extra pulmonary specimens such as pus, lymph node aspirates and wound discharges .Gene X pert negative specimens can be processed in MGIT to recover Atypical Mycobacteria .

Annexures

APPENDIX

1)Modified Ziehl-Neelsen(cold method)

Incrediants

a) Carbolfuchsin

Dissolve 4 g of basic fuchsin in 20 ml of 90-95% ethanol .

Then add 100 ml of 9% aqueous solution of phenol (9 g of phenol in 100 ml of distilled water.

b)Acid- Alcohol

Add 3 ml of concentrated HCL slowly to 97 ml of 90-95% ethanol.

c)Methylene Blue

Dissolve 0.3 g of Methylene blue Chloride in 100 ml of Distilled water.

2)AURAMINE FLUOROCHROME STAIN

a) Phenolic auramine

Dissolve 0.1 g of auramine O in 10 ml of 90-95% etanol&

Add 3 g of phenol in 87 ml of Distilled water

b) Acid Alcohol

Add 0.5 ml of concentrated HCL to 100 ml of 79% alcohol.

c) Potassium Permanganate

Dissolve 0.5 gm of potassium permanganate in 100 ml of Distilled water

3)Lowenstein - Jensens medium

Ethanol	70%
Malachite green solution	2%,
Malachite green dye	2 g

Sterile distilled water	100 ml

PROCEDURE

A) Using aseptic techniques, dissolve the dye in sterile distilled water.

B)place the mixture in the incubator at 37 °C for 1–2 hours or heat in a water-bath at 37°C.

C) Store in dark bottles.

D) This solution is not stable long-term: if precipitation occurs, discard and prepare a fresh solution.

Salt solution

Prepare the salt solution by dissolving the components in distilled water .Autoclave at 121 °C for 30 minutes in a screw-capped bottle.

Hens' eggs

Hens' eggs should be fresh (no more than 7 days old). The eggs should be medium-sized (for balanced proportions of egg white and egg yolk); about 23 eggs will be needed per litre of egg mass.

procedure

- Clean and disinfect the work area.
- Clean the eggs carefully with plain soap and water, soak them in 70% ethanol for 15 minutes and let dry.
- Crack each egg into a small sterile recipient in order to check its freshness.
- Transfer the whole egg into the conical flask with glass beads or magnetic stirrer or into the blender bowl.
- Add sufficient eggs for the volume needed (as indicated in the table below).
- Homogenize the egg mass.
- Add the salt solution.
- Add the malachite green.
- Mix all components gently (to avoid bubbles) to homogeneity.
- Filter through sterile cotton gauze.

- Dispense in 6–8 ml volumes into sterile McCartney bottles or 16 x 125 mm screw-capped tubes.
- Place the tubes on the racks so as to achieve appropriate slopes and inspissate at 80 °C for 45 minutes for three days.

4)Blood agar

Sterile sheep blood 5 ml

Nutrient agar 100ml

Autoclave the nutrient agar base at 121°C for 15 minutes.

Cool to 45-50 ºC and add blood with sterile precautions

and distribute in petri dishes.

5) CHOCOLATE AGAR

Ingredients:

Nutrient agar base 30 gm.

Distilled water 1000 ml.

Sheep blood 100 ml.

Procedure:

1)Dissolve 30 g of Columbia agar base to 1 litre of distilled water in a flask. Heat in a stem sterilizer to dissolve it completely.

2) Adjust the pH to 7.5 – 7.6.

3) Sterilize by autoclaving at 121° C for 15 minutes.

4)Cool to 70° C in a water bath.

5)
Aseptically add 100 ml of sheep blood to it and leave at 70° C for 30 minutes.

6)Mix the blood and agar by gentle agitation from time to time till bloo d becomes chocolates brown in colour. This takes about 10 minutes.

7) Pour as slopes or plates in sterile tubes or sterile Petri dishes.

6) SABOURAUD'S DEXTROSE AGAR Ingredients:

Dextrose 40.0 gm

Neopeptone 10.0 gm

Agar 15.0 gm

Distilled water 1000 ml

Procedure:

1) Mix the ingredients in distilled water by boiling. Adjust pH to 5.6.

2) Sterilize by autoclaving at 115° C for 15 min.

3) Allow to cool to 50° C.

4)
Add Chloramphenicol 1 mg/ml of medium under aseptic precautions.

5) Pour 20 ml amounts in 90mm Petri dish or into 15 ml test tubes.

6)Allow the test tubes to rest at an angle so that agar slopes (slants) are obtained.

7)After the medium solidifies, keep the Petri dish and cotton wool stopped dextrose agar slants in the refrigerator

Proper history will be collected from the patient with the following

PROFORMA:

NAME :

AGE :

SEX :

ADDRESS :

OP/IP NO :

MICRO ID NO :

H/o BCG VACCINATION :

MANTOUX TEST RESULT :

COMPLAINTS :

TREATMENT HISTRORY :

GENERAL EXAMINATION :

SYSTEMIC EXAMINATION :

INVESTIGATIONS

BASIC BLOOD INVESTIGATIONS:

HIV STATUS :

X RAY FINDINGS :

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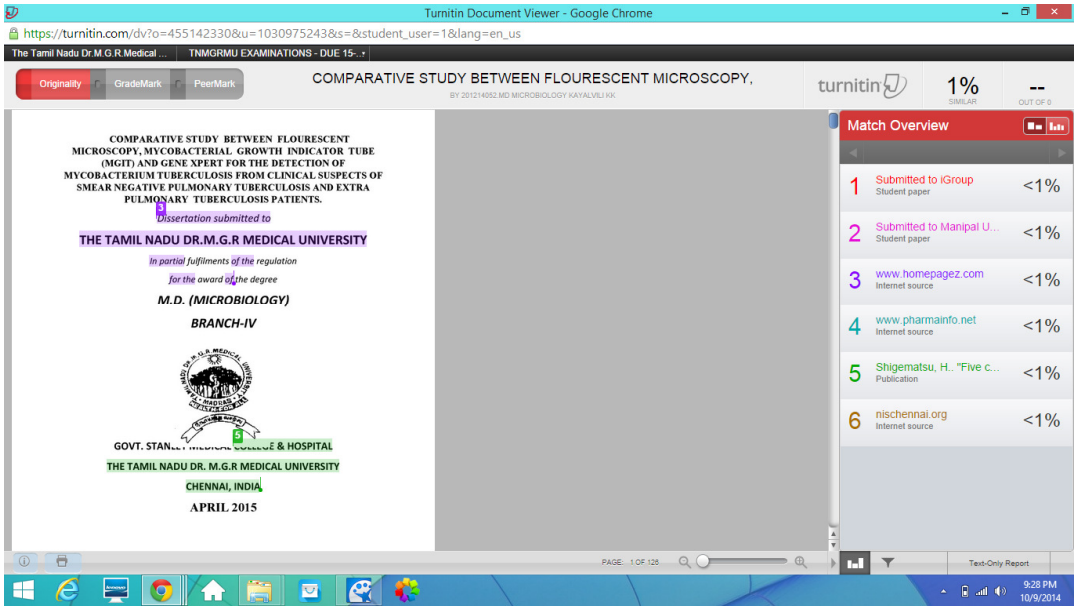
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